

Part I
THE STRUCTURE OF THE
CYTOCHROME C OXIDASE – CYTOCHROME C COMPLEX

Part II
AUTOMATIC DETERMINATION OF
BINDING AND KINETIC CONSTANTS

INAUGURAL-DISSERTATION
zur Erlangung der Philosophischen Doktorwürde

vorgelegt der
Philosophischen Fakultät II
der
Universität Zürich

von
BRUNO MICHEL
von Bönigen (BE)

Begutachtet von
Herrn Prof. Dr. sc. nat. H.R. Bosshard
und Herrn Prof. Dr. Ing. W. Arndt

Zürich 1988
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PREFACE

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PART 1

**The Structure of the
Cytochrome c Oxidase - Cytochrome c Complex
Spectroscopic and Kinetic Characterization**



Summary

There are two major unanswered questions about the mechanism of the electron transfer between mitochondrial cytochrome c and cytochrome c oxidase. The first concerns the reason for the non-linear kinetics of the oxidation of ferrous cytochrome c by oxygen catalyzed by oxidase. The second is the question of how the formation of a protein-protein complex between cytochrome c and oxidase contributes to the acceleration of cytochrome c oxidation by a factor of 10^{13} .

The immediate starting point for the present dissertation was our observation that the absorption spectrum of an oxidase - cytochrome c complex is slightly different from the sum of the spectra of its components. The spectral difference saturates at a ratio of 1 heme c per heme aa₃. These results demand for an explanation of the structural and electronic rearrangements during the binding of cytochrome c to cytochrome c oxidase. A prerequisite for this investigation was the thorough characterization of aggregation state, binding characteristics, and kinetic constants of the oxidase.

Cytochrome c oxidase is a largely asymmetric membrane protein that exists in an equilibrium of monomers and side-by-side dimers. It is only soluble when incorporated in a detergent micelle. A good separation of oxidase into dimers and monomers by gel permeation chromatography was found to be impossible, as shown by experiments and theoretical considerations.

Monomeric beef heart oxidase was obtained by a treatment with Triton X-100 at pH-values above 8.5. The molecular mass of monomeric oxidase was determined by sedimentation equilibrium in solvents of different densities. A molecular mass of 200'000 +/- 20'000 Dal was found for the protein moiety and 120'000 Dal for the detergent micelle.

Binding of cytochrome c to monomeric and dimeric beef heart oxidase perturbed optical spectra in the same way. Similar difference spectra were observed also when cytochrome c was bound to *Paracoccus denitrificans* oxidase. In all cases binding was monophasic, and a stoichiometry of 1 heme c bound per heme aa₃ was found.

Steady-state oxidation of reduced cytochrome c by beef heart oxidase was biphasic at low ionic strengths and at low H⁺ concentrations. In contrast, the two phases merged to one phase at high ionic strengths and at high H⁺ concentrations. Steady-state activity of *P. denitrificans* oxidase at low ionic strength was biphasic. Again, the two phases merged to one phase at high ionic strength. These results proved that biphasic kinetics are a general phenomenon of all oxidases. It can be explained by a model including a conformation change of the oxidase linked to the sequential transfer of two electrons from cytochrome c to oxidase. The results disprove previous models which had been based on two different binding sites and on product dissociation as the rate limiting step of the overall reaction.

CD difference spectra demonstrated that binding of cytochrome c to cytochrome c oxidase induces a change in conformation in both proteins. Most striking was the high specificity of the difference signal; almost no difference signal was observed when cytochrome c was perturbed with solvent, salts and non-specific ligands of cytochrome c, or when oxidase was perturbed in a similar unspecific way. However, there was one exception. The CD difference spectrum induced by a pH increase from neutral to about pH 10, the so called 'alkaline transition', was similar to the CD difference spectrum induced by binding of cytochrome c to oxidase.

Evidence for a change of the heme c electronic structure was obtained from MCD difference spectra due to binding of cytochrome c to cytochrome c oxidase. The maximum of the difference was attributed to a more hydrophobic environment of the heme edge and a less intense electric field across the heme c. Changes of the heme geometry or a better overlap of orbitals are unlikely to be the cause of these MCD differences. MCD and absorption spectra of cytochrome c were sensitive to the overall dipole across the molecule. This observation supports the electron tunnelling hypothesis for the electron transfer (as electron density and therefore tunnelling probability is sensitive to electric field).

Spectroscopic titrations of oxidase with cytochrome c derivatives chemically modified at all of the surface located lysines showed only minor effects of the modification on the affinity, but significant effects on the size and position of the difference spectrum. It seems, therefore, that the mere association between oxidase is very robust. But association has to be followed by conformational rearrangements in order to prepare the associated protein for electron transfer. The rearrangements, in turn, are very sensitive to changes on the surface of either protein.

Very large difference spectra (absorption and MCD) could be detected when cytochromes c with a cleaved peptide bond at position 37, 38, 50, or 55 were bound to oxidase. The 'nicked' derivatives showed significantly lower pK-values for the 'alkaline transition'. When the difference absorption and MCD spectra were corrected for the amount of protein in the alkaline form, the resulting difference spectra looked closely similar to that of native cytochrome c bound to oxidase. Presumably, oxidase is able to impose a 'near native' conformation and electronic structure on the 'floppy' non-covalent derivatives.

Based on the present experimental data and on the observations of Satterlee et al. (1987) and Pielak et al. (1986), the rupture of the Lys 13 - Glu 90 hydrogen bond is proposed to be the key to the 'unlocking mechanism' of cytochrome c. The rupture of the hydrogen bond causes the movement of Phe 82 out of the vicinity of the heme; this is the major structural difference induced in cytochrome c when it binds to the oxidase. Movement of the Phe 82 might be crucial to the process of electron transfer from heme c to heme a of oxidase since replacement of this residue by Ser through site-specific mutagenesis drastically diminishes the rate of electron transfer in a model reaction with cytochrome c peroxidase (Liang et al., 1987).

Zusammenfassung

Zwei wichtige Eigenschaften der Reaktion von Cytochrom c mit Cytochrom c Oxidase sind noch ungeklärt: Die zweiphasige Kinetik der Oxidation von Ferrocytochrom c durch Sauerstoff und die Beschleunigung dieser Oxidationsreaktion um einen Faktor 10^{13} .

Der unmittelbare Anlass für die vorliegende Untersuchung war unsere Beobachtung, dass sich das Absorptionsspektrum eines Oxidase - Cytochrom c Komplexes ein wenig von der Summe der Spektren der Komponenten unterscheidet. Die spektrale Differenz sättigte bei einem Verhältnis von einem gebundenen Häm c pro Häm aa₃. Diese Resultate verlangen eine Erklärung für die Gründe der strukturellen und elektronischen Veränderungen während der Bindung von Cytochrom c an Cytochrom c Oxidase. Eine Voraussetzung für diese Untersuchung war die gründliche Charakterisierung des Molekulargewichtes, der Bindung und der kinetischen Eigenschaften von Oxidase.

Cytochrom c Oxidase ist ein stark unsymmetrisches Membranprotein, das in einem Gleichgewicht zwischen monomerer und dimerer Form vorliegt. Sie ist nur löslich, wenn sie von einer Detergensmicelle umgeben ist. Eine gute Trennung von Oxidase in Monomere und Dimere war unmöglich. Dies konnte durch Experimente und theoretische Überlegungen gezeigt werden.

Rinderherz Oxidase Monomere wurden durch eine Behandlung von Dimeren mit Triton X-100 bei einem pH von über 8,5 erhalten. Das Molekulargewicht der monomeren Oxidase wurde mittels Sedimentations-Gleichgewichts-Zentrifugation in Lösungsmitteln verschiedener Dichte bestimmt. Diese Methode ergab ein Molekulargewicht von 200'000 $\pm$ 20'000 Dal für den Proteinanteil und von 120'000 Dal für die Detergensmicelle.

Die Bindung von Cytochrom c an Cytochrom c Oxidase Monomere und Dimere veränderte die optischen Spektren auf die gleiche Weise. Auch die Bindung von Cytochrom c an *Paracoccus denitrificans* Oxidase ergab ähnliche Differenzspektren. In allen Fällen war die Bindung einphasig und eine Stöchiometrie von einem gebundenen Häm c pro Oxidase wurde festgestellt.

Die Steady-state Oxidation von reduziertem Cytochrom c durch Rinderherz Cytochrom c Oxidase war zweiphasig bei tiefer Ionenstärke und bei hohem pH (tiefe H⁺ Konzentration). Bei höherer Ionenstärke bzw. H⁺ Konzentration vereinigten sich die zwei Phasen. Auch die Steady-state Aktivität von *P. denitrificans* Oxidase war einphasig. Gleich wie bei Rinderherz Oxidase vereinigten sich die zwei Phasen bei hoher Ionenstärke. Diese Resultate zeigen, dass zweiphasige Kinetik eine grundlegende Eigenschaft aller Oxidasen ist. Ein Modell, bei dem der sequentielle Elektronentransfer von zwei Elektronen eine Konformationsänderung in der Oxidase bewirkt, wurde benützt, um diese Beobachtungen zu erklären. Die Resultate widerlegen frühere Modelle, die auf zwei verschiedenen Bindungsstellen basierten und die Produktdissoziation als geschwindigkeitsbestimmenden Schritt annahmen.

CD Differenzspektren zeigten, dass die Bindung von Cytochrom c an Cytochrom c Oxidase eine Konformationsänderung in beiden Proteinen hervorruft. Auffallend war die hohe Spezifität des Differenzsignals; fast gar kein Differenzsignal konnte beobachtet werden, wenn Cytochrom c oder Oxidase mit verschiedenen Lösungsmitteln, Salzen und unspezifischen Liganden perturbiert wurde. Eine Ausnahme war das CD Differenzspektrum, das durch eine pH Erhöhung von neutral zu ungefähr pH 10, dem sogenannten 'Alkalischen Übergang', hervorgerufen wurde. Dieses Differenzspektrum war ähnlich demjenigen, das durch die Bindung von Cytochrom c an Oxidase induziert wurde.

MCD Differenzspektren der Bindung von Cytochrom c an Cytochrom c Oxidase bewiesen, dass die elektronische Struktur geändert wurde. Das Maximum der Differenz wurde der stärker hydrophoben Umgebung der Hämkante und einem schwächeren elektrischen Feld über dem Häm zugeschrieben. Es scheint unwahrscheinlich, dass Änderungen in der Hämgeometrie oder eine bessere Überlappung der Orbitale für diese MCD Differenzen verantwortlich sind. MCD und Absorptions Spektren waren empfindlich auf das totale Feld über dem Molekül. Diese Beobachtung unterstützt die 'electron tunnelling' Hypothese (weil die Elektronendichte von der elektrischen Feldstärke abhängt).

Spektroskopische Titrations von Oxidase mit Cytochrom c Derivaten, die chemisch an allen Lysinen an der Proteinoberfläche modifiziert worden waren, zeigten kleine Auswirkungen auf die Affinität aber grosse Auswirkungen auf die Grösse und die Lage der Differenzspektren. Die blosse Bindung von Cytochrom c an Cytochrom Oxidase scheint sehr robust zu sein. Aber die Bindung muss von konformationellen Änderungen gefolgt werden, die das gebundene Protein für den Elektronentransfer vorbereiten. Diese konformationellen Änderungen hingegen sind sehr empfindlich auf Änderungen auf der Oberfläche von beiden Proteinen.

Sehr grosse Differenzspektren (Absorption und MCD) wurden beobachtet, wenn Cytochrome mit einer gespaltenen Peptidbindung an den Positionen 37, 38, 50 oder 55 an die Oxidase gebunden wurden. Diese gespaltenen Derivate zeigten viel tiefere pK-werte für den 'Alkalischen Übergang'. Wurden die Absorptions- oder MCD Differenzspektren um den Anteil des Proteins in der alkalischen Form korrigiert, so resultierten Spektren, die den Bindungsdifferenzspektren von nativem Cytochrom c ähnlich waren. Man muss daher annehmen, dass die Oxidase diesen 'lockeren' Derivaten eine beinahe native Konformation aufzwingt.

Aufgrund dieser Resultate und den Beobachtungen von Satterlee et al. (1987) und Pielak et al. (1986) wird der Bruch der Lys 13 - Glu 90 Wasserstoffbindung als Schlüssel für die Freigabe des Elektronentransfers vorgeschlagen. Der Bruch der Wasserstoffbindung verursacht eine Bewegung des Phe 82 aus der Nähe des Häms; das ist die hauptsächlich strukturelle Änderung die von der Oxidase im Cytochrom c induziert wird. Die Bewegung des Phe 82 ist vermutlich wichtig für den Elektronentransfer vom Häm c zum Häm a der Oxidase. Diese Vermutung wird untermauert durch die verminderte Elektronentransfer Geschwindigkeit von Cytochrom c auf Cytochrom c Peroxidase, wenn das Phe 82 durch ein Ser ersetzt wird (Liang et al., 1987).

chapter 1

INTRODUCTION

Oxidative phosphorylation is fundamental to all aspects of cellular life in aerobic organisms since it is their main source of useful energy. It is also a particularly challenging research problem, the ultimate solution of which will depend on detailed understanding of electron transport as well as on the molecular organization of the mitochondrial inner membrane.

1.1 *Cytochrome c Oxidase*

It is beyond the scope of this thesis to give a complete summary of the large amount of data published about cytochrome c oxidase. Therefore references will be given to recent reviews on aspects of cytochrome c oxidase research not central to this thesis. As a survey of the entire field of oxidase research the book "*Cytochrome Oxidase : A Synthesis*" by Wikström, Krab and Saraste (1981) is recommended.

Cytochrome c oxidase, the last electron-transporting member of the respiratory chain, catalyzes the reaction:



Due to its central position in the energy metabolism of all respiratory organisms this enzyme has attracted much interest in the fields of bioenergetics and energy metabolism. Its function as an oxidoreductase and as the principal O_2 -reducing enzyme makes it an interesting subject in the fields of biological electron transport and oxygen activation mechanism.

1.1.1 Physical Properties of Oxidase

The cytochrome c oxidase is a transmembraneous protein of the inner mitochondrial membrane. About half of the enzyme protrudes into the aqueous space on the cytoplasmic surface of the membrane. It is this side where the binding of cytochrome c occurs. The matrix side of the enzyme is divided into two domains, giving it the shape of an asymmetric "Y" (Fuller et al., 1979).

Once taken out of the mitochondrial membrane cytochrome oxidase activity depends on a proper surrounding. This means that the membrane has to be 'simulated' by phospholipids or detergents. Phospholipids do not provide an active site (e.g. low affinity site) except for the provision of a suitable three dimensional 'solvent' (Tanford and Reynolds, 1976). The enzyme activity in detergents depends on the proper geometry of the micelle (size of hydrophilic head, length of hydrophobic tail). The highest steady-state activities were observed when the oxidase was solubilized in laurylmaltoside micelles (Rosevear et al., 1980).



Figure 1: Structure of oxidase

Schematic representation of the gross structure and topography of cytochrome c oxidase (from Fuller et al., 1979).

Cytochrome c oxidase contains two A-type hemes (heme a and a₃), and two copper ions. Various spectroscopic methods have been applied to the study of cytochrome oxidase. Vanneste (1966) found that 80% of the absorption at the 605 nm band was due to heme a, whereas both hemes contributed about 50% to the Soret absorption (Figure 3 C). From EPR spectroscopy it can be deduced that the two hemes must have a different configuration (Hartzell and Beinert, 1974). One half of total ferric heme is observed as a low spin signal with typical g-values of 3.2 and 1.5. From MCD spectra Babcock et al. (1976, 1977, 1978) and Thomson et al. (1976) suggested a high spin ferric cytochrome a₃ and a low spin ferric cytochrome a for the oxidized enzyme (Figure 3 A). Near infrared CD and MCD studies have shown separate characteristic transitions for both ferric and ferrous a and a₃ (Eglinton et al., 1980). Very strong magnetic coupling between heme iron and copper of a₃ and Cu_B in the oxidized enzyme suggests that the metals must be very close to each other (Tweedle et al., 1978; Moss et al., 1978). They form an EPR silent S=2 antiferromagnetically coupled binuclear center less than 7 Å apart.

Fluorescence resonance transfer studies (Dockter et al., 1978) indicate a distance of 25 Å between the heme of bound cytochrome c and the nearest heme A. The absence of magnetic interactions between heme a and heme a₃ puts the minimum distance at about 10 Å (Malmström, 1979). The fast electron transfer between the hemes that occurs even

at low temperatures suggests that this distance may not be longer than 25 Å (Chance et al., 1977). Both hemes are oriented with their planes perpendicular to the plane of the membrane. This orientation is observed for cytochrome oxidases from a wide range of organisms. In contrast, the heme c is tilted 70° from the normal of the membrane plane (Vanderkooi and Erecinska, 1975).

Heme a is a six coordinate low spin heme A with imidazole ligands in the fifth and sixth axial position. It is the first electron acceptor within the oxidase. Ferrous heme a₃ is high spin when unliganded, possibly five coordinate with a histidine imidazole in the fifth axial position. In the oxidized enzyme, ferric heme a₃ is high spin and possibly six coordinate. It is coupled via a bridging ligand to Cu_B. In contrast to heme a, heme a₃ reacts with ligands (Ellis et al., 1985). Cyanide reacts with heme a₃ at a slow rate causing a spectral change which is consistent with a high to low spin transition in ferric heme a₃ (Jensen et al., 1984). Binding of ligands to heme a₃ and/or redox changes in one heme cause very small or no changes in the parameters of the companion heme.

The E_m values of the hemes are pH dependent. The pK values of the involved groups must be very similar in the redox-states corresponding to the half reduced enzyme. There are at least two acidic residues in the enzyme, the pK values of which are linked to the hemes' redox-state. The changes in pK seem to occur in response to a change in the redox state of the two heme system as a whole. The high potential redox transition in the heme aa₃ system is strongly pH dependent, whereas the low potential transition is much less pH dependent at pH values around 7 (Wilson et al., 1972). This is a possible explanation for the observed redox anti-cooperativity and might be a key to the understanding of this phenomenon.

Cytochrome c oxidase is a multisubunit enzyme (Wilson and Erecinska, 1979). On SDS gels 13 different polypeptides can be identified (Kadenbach et al., 1983). The sequence of all peptides has been determined (Downer et al., 1976; Buse et al., 1982; Steffens et al., 1979, 1983).

The polypeptides I and II are coded for by the mitochondrial genome, they are common to all oxidases (bacterial and eucaryotic). *P. denitrificans*, yeast, bovine and human subunits exhibit very high homologies (Anderson et al., 1982). They contain almost identical hydrophobic stretches in the sequences of corresponding subunits from different organisms. Subunits I and II lie entirely on the cytoplasmic side of the membrane, but they are still in contact with the lipid bilayer. Although *P. denitrificans* oxidase contains subunits I and II only, it is a fully competent enzyme with all functions of the mammalian enzymes. This finding suggests that subunits I and II are capable of binding all 4 redox centers (Ludwig, 1980; Albracht et al., 1980).

Subunit III contains 7 stretches of almost exclusively hydrophobic residues, which make this protein intriguingly similar to bacteriorhodopsin. This subunit is coded for by the mitochondrial genome too. It can be affinity-labeled on both sides of the membrane as well as inside the membrane and it binds DCCD as does the DCCD binding protein in the F₀ segment of the H⁺ translocating ATP-ase (Casey et al., 1980; Prochaska et al., 1981).

SDS Band No.	SDS (M _r)	Sequence (M _r)		
I	35'300	56'993	(M)	
II	25'200	26'049	(M)	
III	21'000	29'918	(M)	
IV	16'200	17'153		
V	12'100	12'436		
VI	6'700 (3x)	10'670	9'419	8'480
VII	3'400	10'068		
VIII	2'500 (3x)	5'541	4'962	6'244
Sum	150'000	197'933		

(M) : Coded on mitochondrial genome

Table 1: Polypeptide composition of beef heart cytochrome c oxidase

Whether beef heart oxidase preparations lacking subunit III can still pump protons is being disputed (Wikstöm, 1984; Puettner et al., 1983; Puettner, 1984).

Subunits IV to VIII have not yet been assigned a specific function, although removal of some of these peptides results in an enzyme with less or almost no activity. They are coded on nuclear DNA, synthesized by cytoplasmic ribosomes and imported into the mitochondrion (Power et al., 1984). Mutants either lacking mitochondrial protein synthesis or mutants with defects in the synthesis of cytoplasmic oxidase proteins are incapable of building up active cytochrome c oxidase. Clearly, there is cooperation between cytoplasmic and mitochondrial protein synthesis in the assembly of cytochrome c oxidase and other mitochondrial proteins.

1.1.2 Minimum Molecular Weight of Oxidase

There is a great deal of confusion about the molecular weight of oxidase. The terms "monomeric" and "dimeric" are used with different meanings in literature.

Thompson, D.A. et al. (1983) applied beef heart cytochrome c oxidase to a Sephacryl S-300 column in 10 mM Tris-HCl pH 7.8, 100 mM KCl and 0.1% (2mM) laurylmalto-side. The poorly resolved peaks at molecular weights of 650'000, 320'000 and 130'000 indicated different aggregation states of oxidase in exchange with each other. All molecular states were reported to show biphasic steady-state kinetics. Radiation inactivation studies revealed an apparent molecular weight of 70'000 for the functional unit of oxidase.

- Nalecz, K.A. et al. (1983) reported the preparation of a monomeric oxidase showing monophasic steady-state kinetics in 10 mM tris-HCl pH 7.4 and 0.1% laurylmaltoside. The apparent molecular weight of their oxidase was 160'000 as determined by gel filtration.
- Georgevich, G. et al. (1983) could separate a stable monomeric beef heart oxidase after treatment in 320 mM Tris pH 7.8 and 5% Triton X-100. They determined the molecular mass to be between 130 and 160 kDa after correction for bound detergent. Untreated beef heart oxidase was found to have a molecular mass of 325 kDa, whereas monomeric shark heart oxidase had 160 kDa.
- Suarez, M.D. et al. (1984) solubilized beef heart oxidase in laurylmaltoside. The apparent molecular weight measured by gel filtration ranged from 300'000 up to 350'000. The physical molecular mass of oxidase was determined by sedimentation equilibrium experiments in solvents of different densities using mixtures of D₂¹⁸O and H₂O. The molecular mass of 194'000 Dal was consistent with the molecular weight for monomers predicted from their subunit composition. Radiation inactivation experiments resulted in a functional molecular weight of 114'000.
- Saraste et al. (1980) and Love et al. (1970) measured the molecular mass of cytochrome c oxidase by analytical ultracentrifugation. They found a species of 200'000 Dal and termed it dimeric oxidase. Treatment of cytochrome c oxidase at high pH resulted in a smaller species (100'000 Dal) that was termed monomeric.
- Haakvoort et al. (1985) reported to have separated stable monomeric and dimeric forms of oxidase by HPLC. The apparent molecular weight of the monomeric form was estimated to be 200'000 by sedimentation velocity.

Several attempts have been made to split oxidase into smaller subunits without destroying the catalytic activity or the spectroscopic properties. Among the methods used are limited proteolytic digestion (Yamamoto and Okunuki; 1972, Yamamoto and Oori, 1974), chaotropic reagents (Ozawa et al., 1979), and several detergents (Corbley et al., 1984; Azzi et al., 1986).

In order to identify the oxidase as a monomer or a dimer, an accurate determination of the amount of bound detergent is essential. Estimates obtained from UV absorption or radioactive labeling are not precise enough. Determination of molecular weight by gel filtration is unreliable because large asymmetric membrane proteins frequently behave anomalously. In most cases this method overestimates the molecular weight of these proteins (Le Maire et al., 1980). The method of choice for the exact determination of the molecular mass of oxidase is sedimentation equilibrium. When used with solvents of different densities, this method allows the separate determination of the molecular mass of the protein moiety and the bound detergent.

1.1.3 *Paracoccus Denitrificans* Oxidase : A Model System

Cytochrome c oxidases from different bacteria containing only two (Fee et al., 1980; Fukumori and Yamanaka, 1984) or three polypeptides (Gennis et al., 1982; Sone and Hinkle, 1982) have been described. They contain 4 redox-centers and they show similar spectral and kinetical properties. From *P. denitrificans*, the putative ancestor of the mitochondria (Margelis, 1970; John and Whatley, 1975, 1976), an oxidase with only two subunits can be purified (Ludwig and Schatz, 1979). This oxidase closely resembles the mitochondrial enzyme but has only two subunits of M_r 45'000 and 28'000 Dal. Immunological cross-reactivity experiments with antibodies against subunit II from the yeast oxidase reveal close similarity (Ludwig, 1980). Studies with mammalian cytochrome c oxidase implied that proton pumping is linked to subunit III whereas *P. denitrificans* cytochrome c oxidase shows proton pumping without this subunit (Solioz et al., 1982). Because *P. denitrificans* oxidase performs the same function as the mammalian enzyme but is of much simpler subunit composition and truly monomeric (Ludwig et al., 1982), it constitutes a suitable model to study binding and kinetics of the reaction with cytochrome c.

1.1.4 Kinetic Properties of Oxidase

Reduction of O_2 takes place in two concerted two-electron steps, and yet the electron transfer to the a_3Cu_B centre occurs by discrete one-electron steps. This feature is considered important for the coupling of oxidoreduction of cytochrome a to proton translocation. Initially, cytochrome oxidase accepts one electron per a_3 unit in a fast burst phase when reduced cytochrome c and oxidized oxidase are mixed. The electron is distributed about equally between heme a and Cu_A . A possible explanation of this fact is that the acceptance of the second electron can only be achieved with a slow conformational change of the oxidase.

It has been known for a long time (Maly and Lardy, 1954) that cytochrome c oxidase provides for coupling site III between electron transport and ATP synthesis. In his chemiosmotic concept Mitchell (1961) proposed that the generation of an electrochemical proton gradient across the inner membrane is the immediate consequence of mitochondrial electron transfer. Hydrogen ions are of fundamental importance in the oxidase reaction. First, H^+ is required as a substrate in the generation of water following electron transfer to oxygen. Second, cytochrome oxidase catalyzes proton translocation across the mitochondrial membrane (Wikström et al., 1981; Casey et al., 1980; Siegel and Carafoli, 1980; Puettner et al., 1983; Malmström, 1985).

The steady-state kinetics of the reaction between cytochrome c oxidase and cytochrome c have been studied extensively, either polarographically or spectrophotometrically. When studied over an extended range of substrate concentrations, the steady-state reaction was shown not to be first order with regard to substrate. Simple Michaelis-Menten kinetics could not be applied. This led to a large number of attempts to explain this fact:

Ferguson-Miller et al. (1976,1978) proposed three different reactions between cytochrome c and cytochrome c oxidase. The very low affinity reaction ($K_m = 1 \mu M$), the low affinity reaction ($K_m = 0.1 \mu M$) and the high affinity reaction ($K_m = 0.01 \mu M$) observable in polarographic systems only.

Errede and Kamen (1978) proposed 'dependent site' and 'independent site' mechanisms for oxidase. These are extensions of Minnaerts mechanism IV (Minnaert, 1961). The 'dependent site' mechanism assumes a second molecule of cytochrome c reacting with cytochrome c oxidase that has already bound a first cytochrome c in a different way. Both 'dependent site' and 'independent site' mechanisms can be simplified to the rate equation :

$$\frac{V}{E_o} = \frac{P_1 * [S] + P_2 * [S]^2}{1 + P_3 * [S] + P_4 * [S]^2} \quad (2)$$

Speck et al. (1984) proposed a single catalytic site model in the form of :

$$\frac{V}{E_o} = \frac{P_1 * [S] + P_2 * [S]^2 + P_3 * [S]^3}{1 + P_4 * [S] + P_5 * [S]^2 + P_6 * [S]^3} \quad (3)$$

Antalis and Palmer (1982), on the basis of pre-steady-state experiments, suggested a two step reaction at one binding site.

Bisson et al. (1980,1982) demonstrated by cross-linking experiments of cytochrome c with cytochrome c oxidase that one cytochrome c cross-linked per dimer of cytochrome c oxidase was sufficient to completely inhibit the reaction with free cytochrome c. This led to models with negative cooperativity in a dimer.

Wilms, J. et al. (1980) found that the turnover number increased when the pH was lowered from 8.6 to 4.6. They neither observed irreversible denaturation nor a pH optimum. The turnover number was found to be independent of the ionic strength and the type of ions. The pH dependence of the turnover number was described by three sites that can be protonated (pK_a 8.0, 6.5 and 4.8).

Sinjorgo et al. (1984) postulated a single catalytic and a regulatory cytochrome c interaction site. Two consecutive electron transfers are performed on the same reaction site, this was expressed as:

$$\frac{V}{E_o} = \frac{K_{cat} 1 * [S]}{K_m 1 + [S]} + \frac{K_{cat} 2 * [S]}{K_m 2 + [S]} \quad (4)$$

Brzezinsky and Malmström (1986) suggested a steady-state kinetic equation for a proton pump cycle derived by the method of King and Altmann (1956). Their equation is :

$$\frac{V}{E_o} = \frac{a * [S] + b * [S]^2}{1 + c * [S] + d * [S]^2} \quad (5)$$

It should be noted that equations 2, 4 and 5 are of equivalent form and predict the same steady-state behavior of the oxidase reaction.

1.2 Cytochrome c

A c-type cytochrome is defined as a protein having a protoheme IX prosthetic group covalently bound to the polypeptide chain by thioether linkages resulting from condensation of heme vinyl groups with polypeptide cysteine sulfhydryl groups. The chain length varies from 85 to 135 residues, histidine and methionine serve as the axial heme iron ligands, maintaining an oxidoreduction potential from +150 to 380 mV. The available sequence data suggest that the cytochromes c may have descended from a common evolutionary ancestor (Dickerson et al., 1976). They all share the same delocalization of the unpaired electronic spin in the heme plane, an identical geometry of the iron-coordination and very similar intrinsic CD spectra. This is the basis for the conclusion that the molecules still have almost the same structural organization. The chirality of the axial methionine to the heme-iron is R for all mitochondrial cytochromes c, but a few procaryotic cytochrome c exhibit S chirality at this center. An aromatic amino acid at position 82 (numbering of horse sequence) in the primary structure is observed in all cytochromes c with R chiral axial methionine but is missing in the S chiral coordination types (Senn and Wüthrich, 1985). Replacement of this phenylalanine using site directed mutagenesis by any other amino acid including tyrosine results in a loss of negative ellipticity at 417 nm of *Saccharomyces cerevisiae* iso-1-cytochrome c (Pielak et al., 1986). From studies of chemically modified cytochrome c it has been concluded that the conformation of the protein is insensitive to two of the four tyrosyl side chains (Pal et al., 1975), methionine-65 (MacDonald, 1974), and methionine sulfoxide (Pande, 1979).

The iron atom in all cytochromes c alternates between the oxidized or ferric Fe(III) state with a single unpaired electron and the reduced or ferrous Fe(II) state with no unpaired electron. Ferricytochrome c has five distinct pH-dependent conformational states, separated by the dissociation of single protons :

state :	I	————	II	————	III	————	IV	————	V
pK _a			0.42		2.5		9.35		12.76

IV = alkaline conformation of cytochrome c

Cytochrome c is an electron carrying shuttle between cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV). The process is not driven by a decrease of free energy as almost all of the free energy drop occurs within complexes I, II, and IV. The actual pathway of the electron to and from the heme is a source of continued controversy. It is important to remember that the electron channels are not open to solvent but are conduits of hydrophobicity from the heme to the surface. A recent survey on cytochrome c has been published by Margoliash and Bosshard (1983).

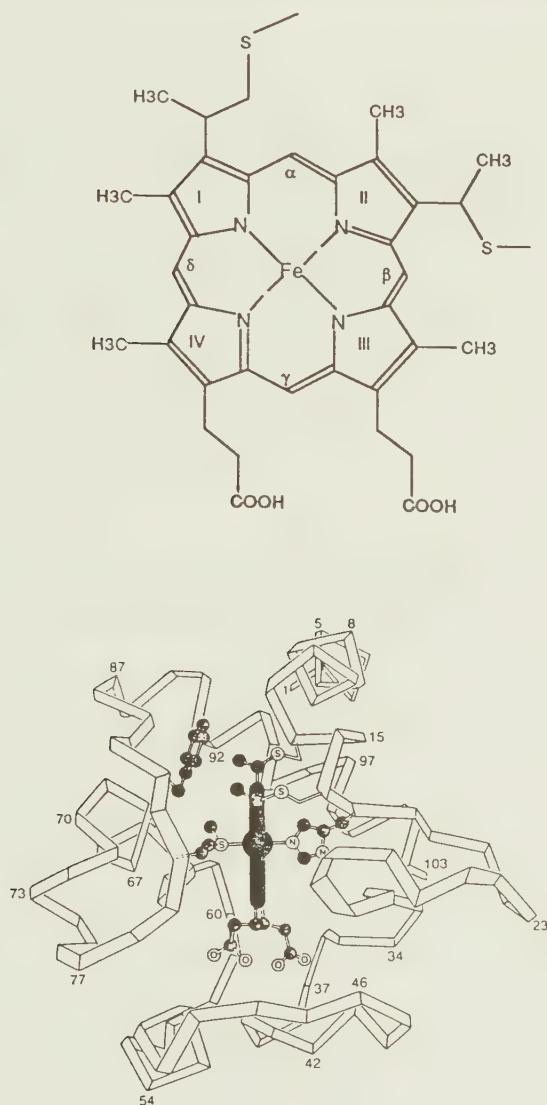


Figure 2: Structure of cytochrome c

Panel A : Heme c.

Panel B: X-ray structure of tuna cytochrome c.

1.2.1 Binding of Cytochrome c to its Redox Partners

Oxidized cytochrome c is a roughly spherical molecule with a diameter between 25 Å and 30 Å. The heme is located in a hydrophobic pocket or cleft, with its edge accessible on the 'front face' of the protein (Dickerson and Timkovich, 1975). A number of lysine residues are surrounding the heme cleft. This ring of positive charges is conserved in all the cytochromes c for which sequence data is available (Dickerson et al., 1976). Modification of any of the positively charged lysines 8, 13, 25, 27, 72, 79, 86 or 87 to an uncharged or negatively charged group affects the activity with oxidase and reductase (Ferguson-Miller et al., 1978; Smith et al., 1977, 1981). The same lysine residues are shielded from acetylation by complexing cytochrome c with either reductase or oxidase (Rieder and Bosshard, 1978, 1980). The same site is involved with other oxidases and reductases. The evolutionary conserved dipolar charge topography of cytochrome c orients the molecule so as to present the binding domain to negatively charged surfaces (Koppenol, 1980).

The three dimensional structures of cytochrome b₅ and cytochrome peroxidase have been determined (Mathews et al., 1971; Poulos et al., 1980). It has been possible, therefore, to fit cytochrome c to these heme proteins by using complementary charged regions. In the hypothetical fit of cytochrome b₅ with cytochrome c the hemes are 8.5 Å apart and parallel (Salemme, 1979; Poulos and Kraut, 1980).

By NMR spectroscopy Satterlee et al. (1987) detected changes in the chemical environment of protons of heme c, His 18, Cys 17, and Met 80 of the cytochrome c molecule in the complex with cytochrome c peroxidase. The most prominent difference was observed for the 3-methyl protons of heme c. These protons are very close to Lys 13 forming a hydrogen bridge in free cytochrome c with Glu 90. Satterlee et al. (1987) suggest hydrogen bonding of Lys 13 with a residue on the surface of peroxidase. This needs a large movement of Lys 13 thus changing the environment of the 3-methyl protons. The significant changes in the Phe 82 and Met 80 resonance indicate a transmission of effects from the surface of cytochrome c to the heme vicinity.

Apart from a slightly less polar environment of the heme in reduced cytochrome c, the structure of reduced cytochrome c is similar to the oxidized form (Takano and Dickerson, 1980). The similarity in the structures of the oxidized and the reduced forms of cytochrome c is consistent with the nearly identical binding constants of the two redox forms for oxidase (Hill and Nicholls, 1980; Peterson, 1978).

1.2.2 Electron Transfer Reaction of Cytochrome c

Although cytochrome c is one of the first proteins to have its sequence determined and its crystallographic structure is available at good resolution, the mechanism of electron transfer through cytochrome c has not yet been clarified. The major obstacle to the investigation of the mechanism is the behavior of its substrate, *the electron*. The electron cannot be

localized precisely due to the Heisenberg uncertainty relation, and it is not possible to trap intermediates. There are two reaction mechanisms suggested for cytochrome c (Marcus and Sutin, 1985).

a) Outer-sphere electron transfer :

Electron transfer can take place via molecular orbitals delocalized throughout the reactant metal complexes. As one pyrrole ring of heme c is accessible on the surface of cytochrome c, a reaction partner could well bring electron conducting functional groups as close to the heme that orbitals can overlap (Tollin et al., 1986).

b) Quantum mechanic tunneling :

The ratio of the squares of the wave function on the outside and on the inside of the barrier represents the probability of electron tunneling :

$$P = \frac{\Psi^2(a)}{\Psi^2(0)} \quad (6)$$

$$P = \exp\left(-\frac{2a\sqrt{2mB}}{h}\right) \quad (7)$$

where a is the thickness in cm, B is the barrier height in Volts, m is the electron mass in g, and h is Planck's constant divided by 2π . The rate of electron escape is proportional to frequency. From a barrier height of 4 eV, a tunneling distance of 15 Å and a frequency of a 5 eV electron in a 7 Å box, a half-time of 2.2 msec for a rectangular barrier can be calculated. This is sufficient to explain electron transfer from cytochrome c to oxidase. The barrier height of 4 eV is consistent with the ionization energies of stacked aromatic side chains (Hopfield, 1974). The extreme sensitivity of tunneling probability to physical proximity and stereochemical matching of macromolecules can explain this 'unlocking mechanism' of cytochrome c.

The tunneling mechanism does not demand for a direct overlap of the orbitals of the reacting complexes since an electron can travel through vacuum due to its characteristic behavior as a wave. The probability of electron tunneling is independent of the energy of the electron and therefore not temperature dependent. Hence, the observed temperature dependence should exclude this model, but tunneling out of a vibronic excited state can introduce temperature dependence as seen for all electron transfer reactions (Hopfield, 1974).

A hypothetical fit between tuna cytochrome c and microsomal cytochrome b_5 by molecular modeling indicated that both mechanisms are possible. Quantum mechanic tunneling could explain electron transfer rates without a conformational change. In contrast, outer-sphere electron transfer needs a deformation of one or both cytochromes.

1.3 Spectroscopic Techniques

1.3.1 Absorption Spectroscopy

The color of the cytochromes is caused by the iron - coordinated protoporphyrin IX. All cytochromes have a characteristic three banded absorption spectrum in the reduced state: The α band around 550-604 nm, the β band around 520-546 nm, and a γ or Soret band around 400-450 nm. The electrons are delocalized among the π -orbitals of the porphyrin ring. The Soret band in the absorption spectrum represents the excitation of delocalized π electrons to unoccupied levels of the porphyrin ring of similar angular momentum (Falk, 1964), a near degenerate allowed $\pi \rightarrow \pi^*$ transition (Adar, 1978). Absorption in the α and β region arises from a comparable excitation to levels of quite different angular momentum. As these transitions are near forbidden, the absorption is weaker. The spectra of the hemes are strongly dependent on the axial ligands. Therefore the heme is a very sensitive marker for any changes of physical properties of the protein.

1.3.2 CD

Circular dichroism is the difference in extinction of left-handed and right-handed circularly polarized light, $\Delta\epsilon = \epsilon_r - \epsilon_l$ where ϵ_r and ϵ_l are the molar extinction coefficients. An alternative way to express circular dichroism is the degree of elliptical polarisation $[\Theta]$ of incident plane polarized light which results from differential absorption of the two components. The two quantities are interconvertible through the relation:

$$[\Theta] \text{ (deg cm}^2 \text{ decimole}^{-1}\text{)} = 2.303 * 4500 / \pi * \Delta\epsilon = 3300 * \Delta\epsilon$$

Theoretical considerations have generally failed to yield quantitative results permitting complete analysis of CD curves. A combination of both the theoretical and phenomenological approach is most suitable for proteins containing extrinsic groups such as heme, metal atom, or FMN.

The CD spectra of all c-type cytochromes are characterized by negative peaks in the region 221-223 nm and 208-210 nm, with positive maxima at 190 and 195 nm. These bands are typical of proteins with low portions of organized structures. The CD spectrum in the aromatic region is composed of two negative extremes at 289 and 282 nm and two positive inflections at 262 and 251 nm. The 262 nm band is ascribed to the integrity of the Met 80-iron linkage (Myer and Pande, 1978). The aromatic region can be used to probe the conformation of several chromophores (tyrosine, phenylalanine, histidine, and tryptophan) (Timasheff, 1970). The ellipticity of the 251 nm band is ascribed to tyrosyl side chains. This band is sensitive to perturbations of the molecule disturbing the heme-tyrosine conformation. The ellipticity of the 252 nm band is caused by phenylalanine.

Free heme exhibits C_{4h} or C_{2v} symmetry and hence no optical activity. If non-identical chromophores are close enough, however, optical activity is generated in both excited states while conserving rotatory strength. For heme c in the protein environment this means that amino acid side chains couple with heme in a non-symmetrical manner. For this reason heme c is a very sensitive marker for protein configuration.

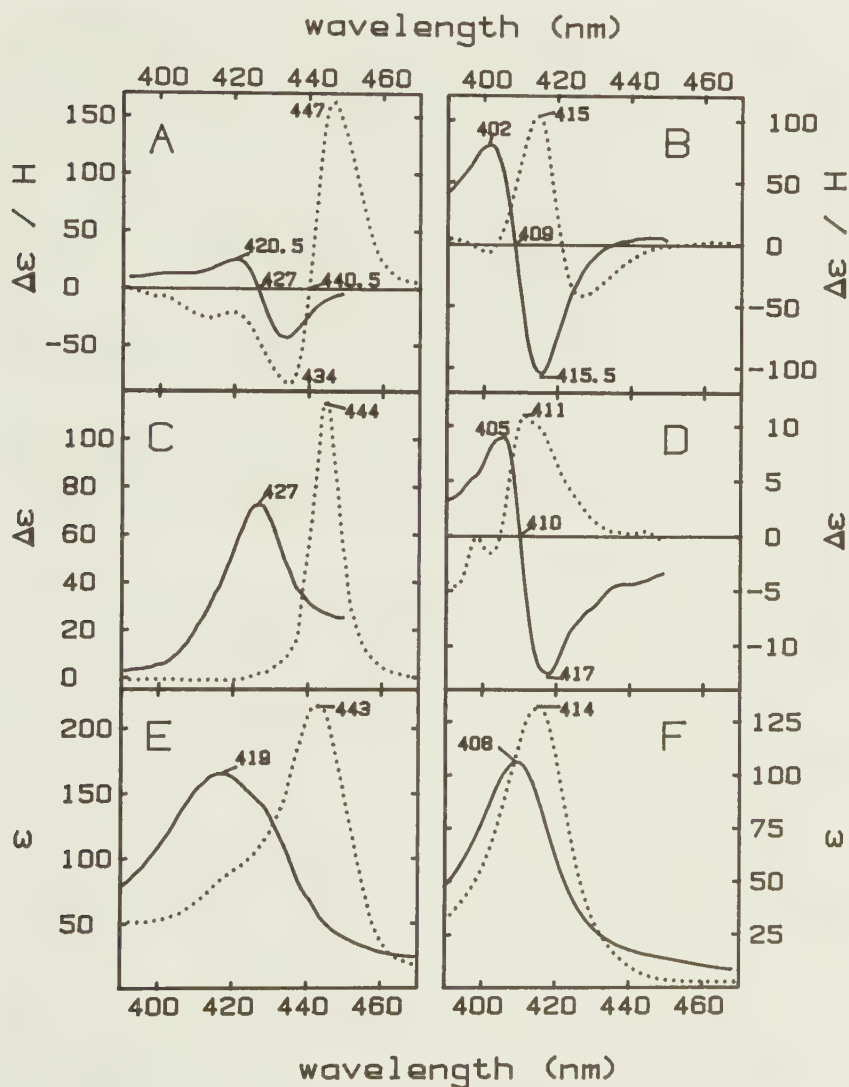


Figure 3: Optical spectra of cytochrome c oxidase and cytochrome c
 Panel A,C,E: cytochrome c oxidase (—, oxidized, ·····, fully reduced)
 Panel B,D,F: cytochrome c (—, oxidized, ·····, reduced)
 Panel A,B: MCD, Panel C,D: CD, Panel E,F: Absorption.

The CD spectrum of horse heart ferricytochrome c in the major heme absorption region is dominated by a pronounced negative peak between 416 and 419 nm, a strong positive peak between 403 and 406 nm and shoulders in the regions 360-370 and 430-440 nm (figure 3 E, solid line). The CD spectrum of the ferrous form exhibits a positive peak centered at 411 nm, a small peak around 400 nm and a negative band at 390 nm (Figure 3 D, dotted line). Since excitation splitting can be excluded for the monomeric, single heme cytochrome c, the symmetry of the heme has to be considerably lower than C_{2v} . This can be due to perturbation of the porphyrin skeleton and the heme iron geometry, or to a coupled oscillator between heme centered π to π^* transitions and allowed π to π^* transitions in a nearby residue, e.g. Phe 82 (Pielak et al., 1986).

The dichroic spectrum of reduced cytochromes in the region 500-600 nm, the α and the β absorption region, is generally highly complex. There are significant differences among various ferrous mammalian cytochromes c, but the bands cannot be assigned to specific groups.

The sign of the Cotton effect at 695 nm has been correlated with the iron-sulfur linkage (Senn and Wüthrich, 1980); a negative sign has been found for cytochromes c with R chirality, and a positive sign represents S chirality of the axial methionine sulfur linkage.

1.3.3 Theory of MCD

MCD is the difference in absorption of left and right circularly polarized light induced by an external, longitudinal (parallel to the direction of light propagation) magnetic field. It is therefore a type of Zeeman experiment where the magnetic field splits or mixes zero-field energy levels.

Unfortunately, MCD has been reported in a wide variety of units. The differential molar extinction coefficient per unit field $\Delta\epsilon_m$, is now more or less generally accepted for expressing MCD. It is defined as

$$\Delta\epsilon_m (M^{-1}cm^{-1}T^{-1}) = \Delta\epsilon/H = \Delta A/(c * l * H)$$

Where $\Delta\epsilon_m = \epsilon_l - \epsilon_r$ and $\Delta A = A_l - A_r$ (the differential absorbance), are induced by the field. H is measured in Tesla (T, 1 T = 10'000 Gauss); c is the concentration in mol l⁻¹ and l the pathlength in cm. Another unit usually found in literature is the molar ellipticity (per unit field), $[\Theta]_m$, which is related to $\Delta\epsilon_m$ as follows:

$$[\Theta]_m (\text{deg cm}^2 \text{ dmol}^{-1} T^{-1}) = 100 * \Theta / (c * l) = 3300 \Delta\epsilon_m$$

MCD can be used to assign bands to particular transitions, characterize ground- and excited-state symmetries and measure intra- and intermolecular interactions. MCD is very sensitive to changes in molecular structure. Although not always well understood in theory, it provides a useful empirical tool for structure determination (Sutherland, 1978; Sutherland and Holmquist, 1980). In principle the MCD dispersion is composed of three terms :

$$\Delta\epsilon = \gamma (-A_1 (\delta f/\delta E) + B_0 f + C_0 f/kT) \beta H \quad (8)$$

called the A, B, and C terms, respectively, where f= form of spectrum, β =Bohr magneton, H= magnetic field (Tesla). The A term will give rise to a derivative shaped dispersion in

an MCD spectrum. Positive A terms have negative lobes at higher energy. B and C terms display line shapes similar to zero-field absorption bands, except for the possibility of both positive and negative sign in MCD bands. A_1 , B_0 , and C_0 are parameters that depend on the electronic dipole selection rules in the magnetic field. They may be distinguished by their dispersion and temperature dependence (Schatz and Caffrey, 1969; Dooley and Dawson, 1984).

1.3.4 A Comparison among Spectroscopic Methods

MCD like CD measures the same physical property (difference between the extinction coefficients of left and right circularly polarized light). The origin of these effects is different. Inherent structural dissymmetries either in the chromophore itself or in the environment in which the chromophore is located give rise to CD effects whereas MCD results from the direct effect of a magnetic field on degenerate electronic levels. Since CD and MCD can be both negative and positive, their spectra show considerably more fine structure than the corresponding absorption spectra. CD is more sensitive to details of micro-environment of the chromophore whereas MCD is more sensitive to changes in the electronic structure which in turn reflects changes in physical structure.

1.4 Aims of the Present Study

Complex formation between oxidase and cytochrome c has been shown to perturb the absorption spectrum of both heme a and heme c. The nature of the difference spectrum has been attributed to a change in the environment of the heme induced by the heme of the redox partner. But the mechanism of this 'induced fit' has not been investigated at all. The difference absorption saturated at a ratio of 1 heme c per heme a₃. Eadie Scatchard transformations of titrations were non-linear. This was explained by : (i) negative cooperativity between monomers in the oxidase dimer, (ii) a cytochrome c bound to a regulating (e.g. low affinity) site, modulating the affinity of the spectroscopic binding site. Other sources for non-linear binding could have been (iii) inhomogeneous oxidase and (iv) oligomeric oxidase leaving part of the binding sites less accessible to cytochrome c (Michel and Bosshard, 1984).

Steady-state turnover of ferrocytochrome c by monomeric oxidase was reported to be triphasic (Ferguson-Miller, 1976,1978), biphasic (Errede and Kamen, 1978), and monophasic (Bolli et al., 1985). A wide variety of models has been presented to explain the biphasic or triphasic steady-state turnover. Several authors explain this phenomenon with a high and a low affinity binding site for cytochrome c on the oxidase, although binding of cytochrome c to oxidase has not always been measured by these authors. It is dangerous to refer to results of binding measurements published by other authors, as different methods of oxidase preparation result in quite different binding and kinetic parameters. It is therefore important that binding and kinetic experiments are performed on the same lot of oxidase and under identical conditions. Moreover, it is essential that the oxidase is thoroughly defined in terms of molecular weight, subunit composition, and phospholipid content.

The molecular weights reported for oxidase competent in electron transfer ranged from 100'000 to 325'000. Different authors use the terms 'monomeric and 'dimeric' in a contradictory manner.

From the points listed above, the following questions arose :

- 1 What is the physical molecular mass of monomeric oxidase ?
- 2 Do monomers and dimers bind cytochrome c in a different manner ?
- 3 Is steady-state activity affected by the aggregation state of the oxidase ?
- 4 How do ionic strength, pH and detergents affect the kinetic constants ?
- 5 Can a difference spectrum be induced by solvents or non- physiological ligands, or is it specific for heme heme interactions ?
- 6 Does oxidase induce conformational changes in cytochrome c ?
- 7 Is the electronic structure of the heme iron affected ?
- 8 Are the difference spectra altered by modification of one or several amino acids of cytochrome c ?

The first topic of this thesis was the determination of the exact molecular mass of the oxidase in different detergents. A monomeric and a dimeric beef heart oxidase had to be prepared under the most similar conditions possible. It was planned to measure the molecular weight of monomeric and the dimeric oxidase by both gel filtration and analytical ultracentrifugation. Comparison of binding of cytochrome c to monomeric and dimeric oxidase should allow to distinguish between explanations (i - iv). Measuring the binding of cytochrome c to the two subunit *P. denitrificans* oxidase was undertaken with the intention of testing the applicability of the binding model to procaryotic oxidases.

The fact that monomeric oxidase shows bi- or triphasic kinetics has prompted investigations of the steady-state turnover of monomeric and dimeric beef heart oxidase as well as *P. denitrificans* oxidase. The influence of ionic strength and pH on kinetic constants and binding might facilitate the selection of a model for the oxidase reaction mechanism.

Since manual assays result in poor accuracy of Michaelis constants, especially with unstable enzymes, an automatic assay mix- and measure robot was developed.

Because binding of cytochrome c to oxidase induces a difference spectrum that is too complex to be fully understood, it was decided to test simpler systems :

- the influence of solvents and different specific non-heme ligands on the absorption spectrum of ferri- and ferrocytochrome c.
- the influence of solvents, ionic strength, and different specific non-heme ligands on the absorption spectrum of fully oxidized and reduced oxidase.

Conformational changes in cytochrome c or oxidase might be detectable by CD measurements. Again, the specificity of these changes might be tested with solvent perturbations. MCD is sensitive to changes in electronic structure of chromophores. Therefore, MCD measurements should reveal the effect of redox partners or non-specific ligands on the electronic structure of heme c. Measuring chemically modified cytochromes c would be helpful to attribute changes of conformation and electronic structure to particular amino acids.

chapter 2

MATERIALS AND METHODS

2.1 Materials

Acetobromo- α -maltose 95% stabilized with 1% barium carbonate, horse heart cytochrome c type III, chicken breast muscle cytochrome c type X, *Candida krusei* cytochrome c type VII, bis-tris (bis(2-hydroxyethyl)amino tris(hydroxymethyl)methane) pKa 6.5, and phosvitin from egg vitellin were obtained from Sigma, St. Louis, MO.

Ammonium molybdate puriss. p.a., ascorbic acid p.a., dichloromethane puriss. p.a., dodecanol puriss., Dowex 1x8 (20-50 mesh, chloride form), EDTA (ethylenediaminetetraacetic acid disodium salt) p.a., ethyl acetate puriss. p.a., ethyl alcohol absolute Ph.Helv., glycine p.a., hexane puriss. p.a., L-histidine puriss., perchloric acid 70% puriss., potassium chloride puriss. p.a., pyridine puriss. p.a., saccharose puriss. p.a., silver carbonate 72.8% purum, sodium chloride puriss. p.a., sulfuric acid, TMPD (N,N,N,N tetramethyl-1,4-phenylene diamine dihydrochloride) purum, and triethylamine puriss. p.a. were obtained from Fluka, Buchs, Switzerland.

Ammonium sulfate fuer biochemische Zwecke, hydrochloric acid 32% pro analysi, methanol pro analysi, potassium hexacyanoferrate pro analysi, silica gel TLC plates (60 F 254 5x7.5 cm), and sodium dihydrogen phosphate pro analysi were obtained from Merck, Darmstadt, Germany.

Tris (tris-(hydroxymethyl) aminomethane) research grade ca.99%, Triton X-100 p.a., Triton X-114 (octylphenol polyethylene glycol ether) pure, Tween-80 (polyoxyethylene sorbitan monooleate) pure, and cholic acid krist. reinst Fp 200-202, was obtained from Serva Heidelberg, Germany.

Drierite 20-40 mesh without indicator was obtained from Aldrich, Milwaukee, WI.

Sephadex G-25 fine and Sephacryl S-300 superfine gels were purchased from Pharmacia fine chemicals, Uppsala, Sweden.

DE-32 (diethylaminoethyl cellulose) anion exchanger and CM-32 (carboxymethyl cellulose) cation exchanger were from Whatman, Springfield, Great Britain.

Ultrogel AcA 54 gel was purchased from LKB, Bromma, Sweden.

UF C 100K ultrafiltration membranes were purchased from Nucleopore Corp. Pleasanton, CA.

Sartorius Minisart NML disposable filter holders pore size 0.2 μ m, diameter 30 mm were supplied by Instrumenten Gesellschaft, Zürich, Switzerland.

The Agla micrometer syringe used in titrations was purchased from Welcome Research Laboratories, Beckenham, England.

2.2 Cytochrome c Oxidase

Electron transfer activity of cytochrome c oxidase was determined spectrophotometrically by measuring the reoxidation of 50 μM dithionite reduced cytochrome c in 100 mM potassiumphosphate pH 6, 0.5 mM EDTA, and 0.2% Tween-80 (Yonetani, 1966). The concentration of oxidase was determined using the extinction coefficients $\Delta\epsilon_{444\text{red.}} = 140$ and $\epsilon_{422\text{ox.}} = 158 \text{ mM}^{-1}\text{cm}^{-1}$ (Van Gelder, 1966).

2.2.1 Preparation of Beef Heart Cytochrome c Oxidase

Beef heart cytochrome c oxidase was prepared according to the procedure of Hartzell and Beinert (1974) and Blair (1967). All purification steps were carried out in a cold room at 4°C. The last pellet of the ammonium sulfate fractionation was homogenized in a minimal volume of 10 mM tris-HCl pH 7.4, 15 mM NaCl and stored at -80°C.

The most critical step in this preparation is the differential solubilization of the bc₁ complex and other membrane proteins with Triton X-114. The oxidase containing particles sediment in 2.5 mg Triton X-114 per mg of protein within 14 hours at 9500 g. If the ratio of Triton to protein or the centrifugal force is different, the purification inevitably fails, and has to be started from the beginning.

Another pitfall is a lack of detergent during the repetitive ammonium sulfate precipitations. This can be prevented by addition of small amounts of Tween-80 (not laurylmaltoside) to the third or fourth homogenizing buffer.

a) Change of detergent

The last homogenate was diluted 30 times in 10 mM tris-HCl pH 7.4, (and the desired detergent), applied to a DE-32 column (1.5 * 9 cm), washed with 10 times the column volume, and eluted with the same buffer containing 200 mM NaCl. The eluted oxidase was pooled, concentrated to about 120 μM in 10 mM tris-HCl pH 7.4, 15 mM NaCl, and 0.1% laurylmaltoside or 0.2% Tween-80 by ultrafiltration using an UF C100 membrane, and stored at -80°C.

b) Alkali treatment of oxidase

Cytochrome c oxidase was incubated in 10 mM tris-HCl pH 10.5 for 6 hours (Chan et al., 1970, Love et al., 1970). It was then applied to Sephacryl S-300 in order to check the molecular weight distribution. The proportion of monomeric oxidase was significantly increased compared to untreated oxidase, but this preparation was still inhomogeneous.

c) Alkali and Triton X-100 treatment of oxidase

Monomeric beef heart cytochrome c oxidase was prepared according to Georgevich et al. (1983). The last homogenate from the ammonium sulfate precipitation was incubated with an equal amount of a buffer containing 10% Triton X-100, 640 mM tris-HCl pH 8.5 for two hours on ice. The probe was diluted 30 times with distilled water, applied to DE-32 cellulose, as in a) above, washed with 20 column volumes of buffer, eluted and concentrated in the same way.

d) Proteinase treatment of oxidase

Proteinase treated beef heart cytochrome c oxidase was prepared according to Yamamoto and Orii, 1974. Cytochrome c oxidase (50 μ M) was dialysed against 50 mM sodium phosphate pH 7.8 and 0.05% cholate. The cholate concentration was then increased to 0.5% and chymotrypsin was added (1/20 w/w). Cytochrome c oxidase was incubated for 3 hours at 20 °C, then the proteolytic fragments and residual chymotrypsin were separated from the oxidase on an AcA 54 column in 10 mM tris-HCl pH 7.4, 15 mM NaCl, and 0.2% Tween-80.

e) Resolution into subunits

Cytochrome c oxidase was resolved into subunits according to Corbley and Azzi (1984). The enzyme was dialyzed against 20 mM tris-HCl pH 7.4, 0.05% Triton X-100 and 0.0005% lithium dodecylsulfate. The enzyme was then incubated in 0.2% lithium dodecylsulfate on ice and the subunits were separated on a Sephadex G-200 column in 10 mM tris-HCl pH 7.4, 0.05% lithium dodecylsulfate, 0.05% Triton X-100. Two peaks were pooled and concentrated by ultrafiltration in 10 mM Tris HCl, 0.2% Tween-80, and 15 mM NaCl. The first peak was shown to consist of subunit I and II, the second peak contained subunits I, II and III.

2.2.2 Paracoccus denitrificans Oxidase

Paracoccus denitrificans oxidase was a gift from Dr. B. Ludwig (University of Luebeck). It was isolated as described by Ludwig and Schatz (1980). In addition to the standard purification procedure the sample was purified by affinity chromatography on a cytochrome c column in 10 mM tris-HCl with no additional detergent apart from small amounts of Triton/ cholate still present. The heme to protein ratio was 34.3 nmoles heme a per mg of protein. The oxidase was used without further purification for binding experiments and kinetics. Concentrations of *P. denitrificans* oxidase were determined taking the absorbance at 424 nm minus the absorbance at 480 nm and an extinction coefficient of 60.1 mM⁻¹cm⁻¹ (Ludwig and Schatz, 1980).

2.3 Cytochrome c

2.3.1 Horse Heart Cytochrome c

Cytochrome c, Sigma type III was used without further purification. The concentration was determined from the isosbestic absorbance at 410 nm = 106.1 mM⁻¹cm⁻¹ (Margoliash and Frohwirth, 1959).

Cytochrome c was reduced with an excess of sodium dithionite. Unreacted sodium dithionite was separated from cytochrome c on a small Sephadex G-25 column (0.5 * 2cm). The elution speed was increased with a table-top centrifuge as described by Bresnick (1982).

2.3.2 Modified Cytochromes c

Chicken flight muscle cytochrome c and *Candida krusei* cytochrome c were used without further purification. Their concentration was determined from the isosbestic absorbance at 410 nm using the same extinction coefficient as for horse heart cytochrome c.

Horse heart cytochrome c modified at all lysine residues and non-covalent complexes were kindly provided by Drs. C.Wallace and A. Proudfoot from Geneva. For the modified cytochromes c the wavelength of the maximal absorption in the Soret region was determined and the concentration calculated from the absorption at this wavelength ($\epsilon=106.1 \text{ mM}^{-1}\text{cm}^{-1}$).

2.3.3 Chloramine-T Modified Horse Heart Cytochrome c

Chloramine-T (sodium-N-chlorotoluene-p-sulfonamide) is a mild oxidizing reagent known to specifically modify methionine and cysteine at pH 7-8.5. As cytochrome c contains no free cysteine, only methionine 65 and methionine 80 are oxidized. The reaction can be monitored by the decrease of the absorption at 695 nm. This band shows the Met-80-Fe bond that is destroyed by chloramine-T.

Cytochrome c was modified in 50 mM tris-HCl pH 8.5 by a fourfold excess of chloramine-T as described in Pande (1979). The mixture was desalted and the different modified derivatives were separated and rechromatographed on a CM-32 column ($0.9 \times 25 \text{ cm}$) with a linear gradient of 0-500 mM KCl. Four different peaks were collected, rechromatographed on a second CM-32 column of the same size, concentrated by ultrafiltration and brought into a buffer of 10 mM tris-HCl pH 7.4 and 15 mM NaCl. The concentration of the F-II and F-III derivative was determined using the extinction coefficients determined by Rechsteiner (1986): F-II : $\epsilon_{400} = 124.5 \text{ mM}^{-1}\text{cm}^{-1}$, F-III : $\epsilon_{400} = 126 \text{ mM}^{-1}\text{cm}^{-1}$.

2.4 Spectroscopic Methods

2.4.1 CD and MCD Spectra

a) Absolute spectra

CD and MCD spectra were recorded on a Jasco 500C recording polarimeter equipped with a electromagnet of 1.5 Tesla (15'000 Gauss). The spectra were recorded at $25 \pm 1^\circ \text{C}$ in a cuvette with a pathlength of 1 cm. Natural CD was removed from MCD by subtracting a measurement with the magnetic field parallel to the light beam and a measurement with the magnetic field antiparallel to the light beam. Scan speeds were 20 or 50 nm/min and integration times 1 or 0.5 seconds. Spectra were stored and calculated on an Epson QX computer attached to the polarimeter. CD and MCD of buffer was recorded under the same conditions and subtracted from the spectra. Samples used to measure CD and MCD spectra had a maximal absorption of 1 (± 0.1), to optimize the quality of the signal in the region of interest.

b) Difference spectra

Difference spectra were recorded in a tandem cuvette with an optical pathlength of 2×0.4375 cm. The cuvette was filled with 1 ml of probe A in compartment A and 1 ml of probe B in compartment B. First, a spectrum with the magnetic field parallel to the light beam was recorded (spectrum 1). The scan speed was 10 nm/min, and 50 nm were scanned 6 times totalling 30 minutes measuring time; integration time was 2 seconds. Then the field vector was reversed and another spectrum was taken at identical scan speed (spectrum 2). Second, probes A and B were mixed gently by tilting the sealed cuvette about 9 times. Care was taken to prevent foaming and to keep the compartments equally filled. The next spectrum was measured with the magnetic field vector parallel to the light beam (spectrum 3). The field vector was again reversed and another spectrum was recorded (spectrum 4).

The MCD difference spectrum was calculated as follows:

$$\text{Difference} = (\text{spectrum 3} - \text{spectrum 4})/2 - (\text{spectrum 1} - \text{spectrum 2})/2$$

The CD difference was calculated as follows:

$$\text{Difference} = (\text{spectrum 3} + \text{spectrum 4})/2 - (\text{spectrum 1} + \text{spectrum 2})/2$$

2.4.2 Spectroscopic Titrations

Difference spectra using tandem cuvettes have been performed for the cytochrome c - cytochrome c peroxidase complex (Erman and Vitello, 1980), for the cytochrome c - cytochrome b₅ complex (Mauk and Mauk, 1982 a), and for the cytochrome c - methemoglobin complex (Mauk and Mauk, 1982 b). Measuring the difference spectra at different ratios of cytochrome c to ligand resulted in large noise that prevented an analysis of the dissociation constant. The noise was due to the repositioning of the tandem cuvette in the light beam after the mixing of the two compartments and to the poor longtime stability of the oxidase absorption. Therefore, spectroscopic titrations were performed on a HP 8451 diode array spectrophotometer equipped with an Agla syringe. A computer controlled stepper motor drive was added in order to increase reproducibility, precision, and speed of titrations. For technical details, see Part Two.

All buffers and enzyme solutions had to be degassed and filtered through a 0.2 μm filter prior to measurements. Care was taken to keep every part perfectly dust free. The measurements consisted of the following steps :

After the photometer had been allowed to warm up for at least 1 hour, total light intensity (I_0) and stray light (I_s) were measured. After that, the Agla syringe was filled and equilibrated out of a perfectly homogeneous and dust free solution of 20-50 μM cytochrome c.

A small magnetic stirrer was inserted into a 3 ml Hellma cuvette, 2000 $\pm$ 0.2 μl of buffer was placed in the cuvette on a Mettler analytical balance.

The cuvette was fixed in a thermostated cell holder with a turbine driven magnetic stirrer.

The syringe tip was cleaned, submerged about 2-3 mm in the buffer in the cuvette and cytochrome c was added in steps of 10 μl . After 15 seconds of mixing a spectrum was accumulated for twice 5 seconds, the stability was checked, and if stable the spectrum was stored.

After the first series had been finished, the Agla syringe was refilled and 2000 $\pm$ 0.2 μl of 4 μM cytochrome c oxidase was inserted into the same or a perfectly matched 3 ml cuvette again equipped with a magnetic stirrer.

A baseline with buffer was measured, then the cuvette with the oxidase was fixed in the cell holder and a spectrum of the oxidase was taken and corrected for stray light.

The needle of the refilled syringe was cleaned and submerged in the cytochrome c oxidase solution.

Cytochrome c was added in the same aliquots as in the first series, the solution was allowed to mix for the same period of time, and a spectrum was measured twice for 5 seconds.

All spectra were corrected for stray light as described in Part Two of this volume.

The 40 difference spectra were stored and a titration curve was calculated and printed.

2.5 Determination of Kinetic Constants

Michaelis constants and first order rate constants were determined with the "Assayomate" in conjunction with a HP 8451 spectrophotometer and a HP-150 personal computer. The "Assayomate" is a self-built device capable of mixing assays of variable substrate concentrations. The assay mixture is transferred to a 1 cm flow cell placed in the HP 8451 photometer with a dead time of 200 milliseconds. The time course of the optical density is measured at two different wavelengths simultaneously. A derivative of optical density versus time is calculated and the rate is averaged over a time window that can be specified during setup. The derivative is checked for excess noise and nonlinearity and stored if it fulfills the specified requirements. For each substrate concentration this procedure is repeated from two to ten times. The different experiments are averaged and checked for reproducibility. If the standard deviation was small enough, the result was stored. For a single Michaelis constant between 5 and 40 different substrate concentrations were mixed and measured in this way. After successful termination of the measurements the results were converted into turnover number and stored on disc. The data was transformed into double reciprocal form (Lineweaver Burke), a linear regression was performed, and maximal velocity, Michaelis constant and correlation coefficient were printed (see Part Two of this volume).

2.5.1 Setup for Assays

Buffer of the desired pH, ionic strength and detergent was sucked into syringe 1 of the "Assaymate". Cytochrome c was reduced as stated above, stock solutions of 100 to 300 μM were prepared and placed in syringe 3. Cytochrome c oxidase was diluted to 50 nM and stored on ice for up to 1 hour. Immediately prior to measurements the oxidase was filled into syringe 2 of the "Assaymate" and thermostated to the desired temperature. For the experiments in this work, cytochrome c concentrations ranged from 0.15 to 30 μM . The oxidation of cytochrome c was measured simultaneously at 418 and 550 nm. A Michaelis constant was calculated from 20 different substrate concentrations measured 3 or 4 times each.

Statistical information on the accuracy of the Michaelis constants was obtained by repeating the measurement described above from two to six times. Possible linear inactivation of oxidase during the measurement could be compensated by measuring the series with increasing and decreasing substrate concentrations.

2.5.2 Data Analysis

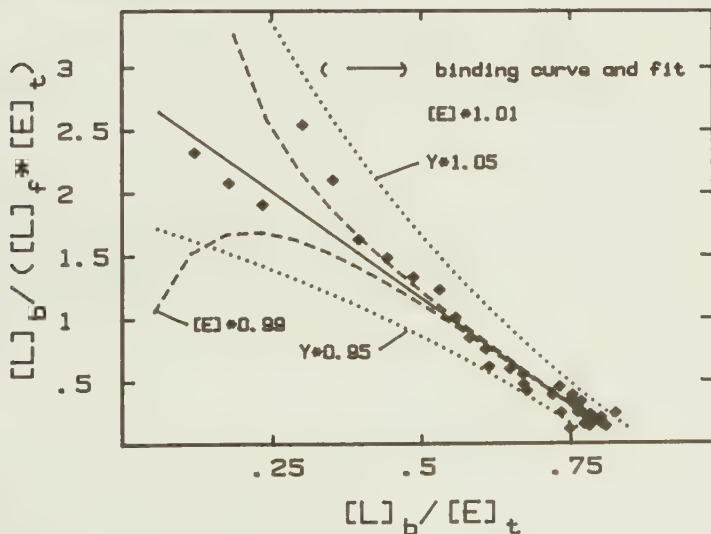


Figure 4: Errors in Scatchard plots

(♦) sample binding data, (—) optimal fit, (·····) $\pm 5\%$ error in the binding signal, (-----) $\pm 1\%$ error in enzyme concentration.

As computers have become cheaper and easier to handle, fitting of data to models has become a useful tool for the biochemist (Sagnella, 1985). A large number of fitting programs using different fitting procedures are available (Duggleby, 1981, 1985; Knack and

Röhm, 1981a, 1981b; Clealand, 1979; Mannervik, 1982). Common to all these programs is that they behave like a black box : they are fed with a number of digits (first estimates), they do a fitting procedure and then print out a set of numbers that have to be converted into the actual parameters. The only information about the quality of a fit is the mean root square. But this value is of little help to the selection of the best among different models. The least square optimization procedures may be caught in a local minimum or the optimization is stopped prematurely because of very slow convergence. A mathematically correct solution of an equation does not always lead to physically meaningful parameters. Furthermore the implementation of new models is difficult for the average biochemist, as equations with partial derivatives have to be deduced, programmed and tested.

a) Linearization methods

Conventionally, different mathematical transformations have been used to analyze data from graphical representation. Mostly, the aim of these transformations was a linearization. But as models have become more complex, the user has to be aware of the limitations of these plots, as errors in some segments of the curves are magnified by these transformations. This can lead to wrong answers. The Eadie Scatchard and Eadie Hofstee transformations are used in this volume to visualize and check the results of the mathematical fit routines. Figure 4 shows a set of data, a fitted curve and a $\pm 5\%$ error envelope that visualizes the different magnification of the error in different segments of the curve (Naquil, 1986).

b) Direct curve fit

In view of the drawbacks of both the linearization methods and the direct curve fit programs available, a fit program was developed in order to analyze the binding experiments and non Michaelis Menten kinetics. As data was already available in digital form, a data handling program was developed. The titration curve is read into a work space called "register". The absolute noise is read into another register. In the fitting program a fixed model can be selected or an own model can be entered for example :

$R = C_1 S / (C_2 + S)$ for simple Michaelis Menten kinetics.

C_1 / C_2 : these are the parameters searched for.

R : dummy argument telling the system to do a regression.

S : set of substrate concentrations (variables).

During the fitting process all intermediary results are being displayed. First a large grid, covering the entire meaningful area is generated. This prevents the routine from being caught in local minima. The fit routine then tries to improve the first estimates by performing a binary grid search. If the values are within the area of such a field, the grid is made finer, until the final resolution is achieved. After successful termination of the fit, a theoretical curve is simulated with the result of the fit. The simulation can then be displayed together with the data in Scatchard plots or any other transformation. The user is thus able to check the quality of the results by the conventional method. (For more information about these programs see Part Two).

2.6 Miscellaneous Procedures

2.6.1 Gel Permeation Chromatography

Sephacryl S-300 columns (2.4 * 80 and 0.9 * 60 cm) were calibrated with the following proteins of known molecular weight :

Protein	M_r (kdal)	Partition coefficient
thyroglobulin	669	0.10
ferritin	440	0.22
alcohol dehydrogenase	141	0.43
BSA	68	0.57
chymotrypsinogen	25	0.82
cytochrome c	12.5	0.96

Table 2: Calibration of S-300 columns

A partition coefficient of 0 corresponds to an apparent molecular weight larger than 2'000'000 and a partition coefficient of 1 to an apparent molecular weight smaller than 5'000.

2.6.2 Analytical Ultracentrifugation

The sedimentation equilibrium distribution of a particle sedimenting at a given angular velocity depends on its mass, its volume in solution, and the density of the solution. It can be described by the equation:

$$M_r(1-\bar{v}\rho) = \frac{2RT}{\omega^2} \frac{d \ln c}{dr^2} \quad (9)$$

where M_r is the molecular mass of the sedimenting particle, $\bar{v}$ its partial specific volume, ρ the density of the solvent, R the gas constant, T the absolute temperature, ω the angular velocity, c the concentration of the particle, and r the distance from the axis of rotation.

The physical molecular weight of the oxidase - detergent complex was determined using a Beckmann L5-75B ultracentrifuge equipped with a UV scanner. Protein concentration was measured using a 278 nm filter. Sedimentation equilibrium runs were performed in a AN-G rotor at 4 °C. 1.5 μ M oxidase in 10 mM tris-HCl, 40 mM NaCl, and 0.1% laurylmaltoside was centrifuged at 14'000 rpm for 5 hours, then the speed was lowered to 9'000 rpm. The concentration distribution of oxidase was scanned after 19 and 34 hours and the natural logarithm of the absorption was plotted vs. the square of the radius (m^2). The molecular mass was determined from the slope of a line drawn through the data points.

When the sedimenting particle is a protein-detergent complex, the contribution of its individual components to the sedimentation equilibrium distribution can be separated (Reynolds and Tanford, 1976).

$$M_p (1 - \bar{v}_p \rho_s) + M_d (1 - \bar{v}_d \rho_s) = \frac{2RT}{\omega^2} \frac{d \ln C}{dr^2} \quad (10)$$

Where M_p and $\bar{v}_p$ are the molecular mass and the partial specific volume of the protein moiety and M_d and $\bar{v}_d$ the total molecular mass and partial specific volume of the detergent micelle associated with the protein. If the solvent has the same density as the detergent, the detergent no longer affects the sedimentation equilibrium of the protein.

$$M_p (1 - \bar{v}_p \rho_s) = \frac{2RT}{\omega^2} \frac{d \ln C}{dr^2} \quad (11)$$

M_p can be determined when the density of the detergent is known. The dependence of the apparent molecular weight M_r on solvent density is determined and extrapolated to the density of the detergent (Tanford and Reynolds, 1976). The molecular mass of the detergent and the oxidase solutions in D_2O had to be corrected for proton-deuterium exchange (Edelstein and Schachman, 1967). The correction in 100% D_2O is $K = 1.0155$ for protein and $K = 1.0137$ for laurylmaltoside (Suarez et al., 1984).

2.6.3 Phosphorus Determination

Inorganic phosphorus was determined with the ascorbic acid method of Ammon and Hinsberg described by Chen et al. (1956). The procedure was adapted for the determination of small amounts as follows :

50-250 μ l of sample was ashed in borosilicate glass tubes with two drops of conc. sulfuric acid and heated in a 300°C sand bath until white foam appeared. Carbon was oxidized by 1 drop of 72% perchloric acid and heated until the sample was clear. As the sample was diluted to 2.5 ml only, the acids had to be neutralized with 2 drops of 6N NaOH. 1 ml out of the 2.5 ml was mixed with 1 ml of reagent C (10 ml 6N sulfuric acid, 10 ml 25% ammonium molybdate, 10 ml 10% ascorbic acid and 20 ml of water, prepared fresh daily). The sample was heated for 2 hours to 37°C, the color was measured at 820 nm and compared with standards of inorganic phosphorus treated the same way.

2.6.4 Protein Determination and Polypeptide Analysis

Protein concentrations were determined with the Coomassie-blue assay (Bio-Rad), using BSA as a standard.

The subunit composition of oxidase was examined by SDS-PAGE using the standard procedure of Laemmli (1970). Gels were stained with Coomassie brilliant blue. The molecular weight of peptides was estimated from the comparison with standards of known molecular weight.

Protein	M_{app}	$\ln (M_{app})$
phosphorylase B	97'000	11.482
BSA	67'000	11.112
c AAT	47'000	10.758
m AAT	45'000	10.714
carboanhydrase	31'000	10.342
myoglobin	17'500	9.770
cytochrome c	12'500	9.433

Table 3: Marker proteins on SDS gels

2.6.5 Laurylmaltoside Synthesis

a) Synthesis

Laurylmaltoside was synthesized according to the procedure described in Rosevear et al. (1980), with minor modifications described in Weber (1986) and VanAken et al. (1986). 19.9% of the acetobromo- α -maltose could be converted to the pure product (literature: 25%). Chromatography on Dowex is used to purify the detergent obtained by reacting acetobromo- α -maltose with dodecanol.

b) Preparation of Dowex 1x8 column

1 kg of Dowex 1x8 (mesh 20-50, chloride form) is treated with 5 l 2N NaOH, then washed 5 times with 5 l deionized water. Metal ions and impurities are removed by treatment with 5 l 2N HCl, after 15 minutes the resin is again washed 5 times with 5 l deionized water and brought into the hydroxide form by treating it 4 times with 5 l 2N NaOH (the selectivity for hydroxide is 20 times weaker than for chloride). NaOH is removed by washing the resin 10 times with 5 l of deionized water or until the pH is neutral. After this the resin is brought into methanol and poured into the column.

b) Thin layer chromatography

Thin layer chromatography is used to monitor the reaction.

solvent system I : ethylacetate / hexane (1:1 v/v)
 laurylmaltoside peracetate $R_f = 0.7$
 maltose $R_f = 0.4$

solvent system II: ethylacetate / methanol (4:1 v/v)
 laurylmaltoside $R_f = 0.43$.

TLC plates were developed by spraying with 2 N sulfuric acid and heating to 250°C on a hot plate (or to 90°C in an oven).

chapter 3

RESULTS

3.1 Properties of Beef Heart Cytochrome c Oxidase

The oxidase usually had a heme content of 9 nmoles heme A / mg of protein. It contained 18 ± 1 moles lipid phosphorus per mole of heme aa₃. The ratio of $\epsilon_{444\text{red}} / \epsilon_{422\text{ox}}$ was 1.37 (Figure 5), and the ratio of $\epsilon_{280} / \epsilon_{420}$ was 2.5. The yield from one beef heart varied from 250 to 700 nmoles heme A.

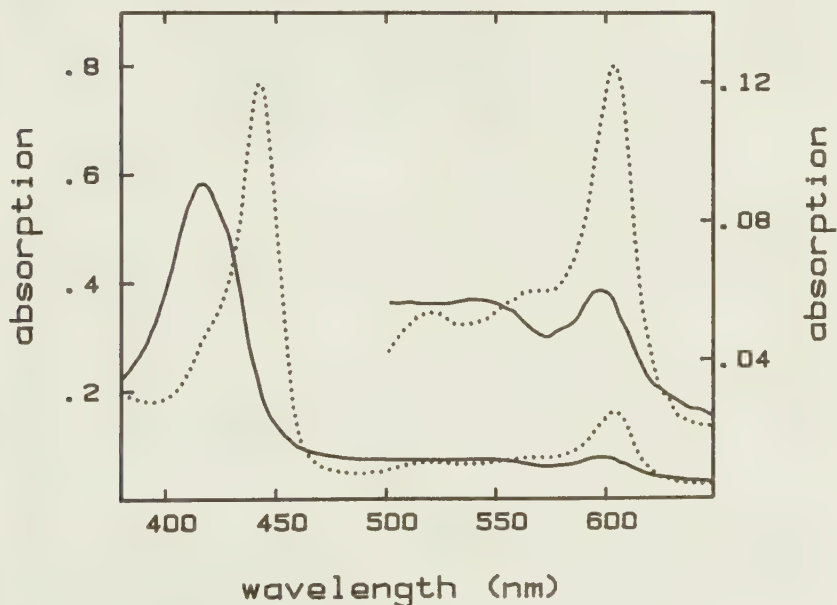


Figure 5: Spectrum of beef heart oxidase

On SDS-PAGE 13 subunits were identified (Figure 6). SDS-PAGE was used to determine the molecular weight of the subunits. A set of marker proteins of known molecular weight was used to calibrate the gel (see Methods). Assuming that the oxidase contains one copy of each subunit and that each is a true subunit, a minimal molecular weight of 190 kDa was estimated (Figure 6).

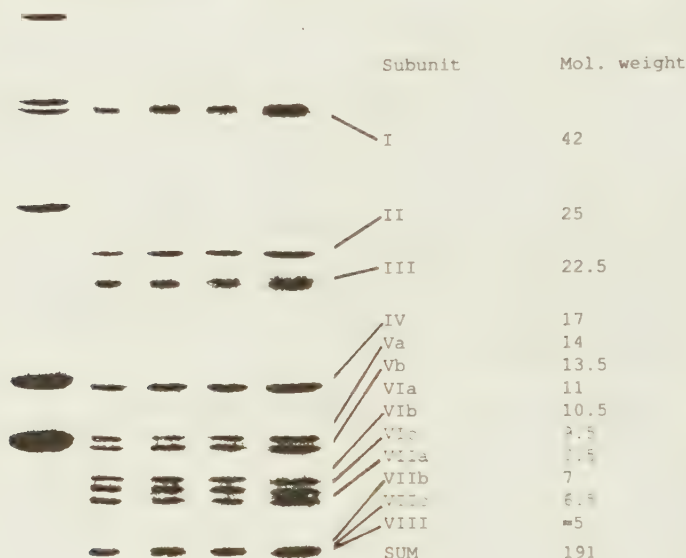


Figure 6: SDS-PAGE of beef heart oxidase

Three different methods have been used to equilibrate the enzyme with the desired non-ionic detergent.

a) dialysis

b) gel filtration on an Aca 54 column

c) ion exchange chromatography on CM-32 cellulose

The last method is most efficient in removing large aggregates of oxidase. For that reason, the oxidase solutions prepared by this method are much less turbid and best suited for spectroscopic titrations.

3.1.1 Molecular Weight of Oxidase

Cytochrome c oxidase was equilibrated into 0.1% laurylmaltoside, 100 mM KCl, 10 mM tris-HCl pH 7.4, 1 mM EDTA by means of a Aca 54 column, and applied to a calibrated Sephacryl S-300 column (2.4 * 80 cm). The elution was monitored at 280 nm (Figure 7 A, solid line). The oxidase concentration in the collected fractions was measured using the absorption at 420 nm (Figure 7 A, dotted line). The collected fractions were assayed for steady-state activity of oxidase as described in Methods. The elution profile was closely similar to the elution profile published in Thompson and Ferguson-Miller (1983).

Twelve pools in the molecular weight range from 1 Mdal to 40 kDa were formed (Figure 7 A). For all pools SDS-PAGE showed the same major bands as observed for native oxidase. Pools #3, #6 and #9 were rechromatographed on an analytical S-300 column in the same buffer (Figure 7 B). Analytical ultracentrifugation was performed on

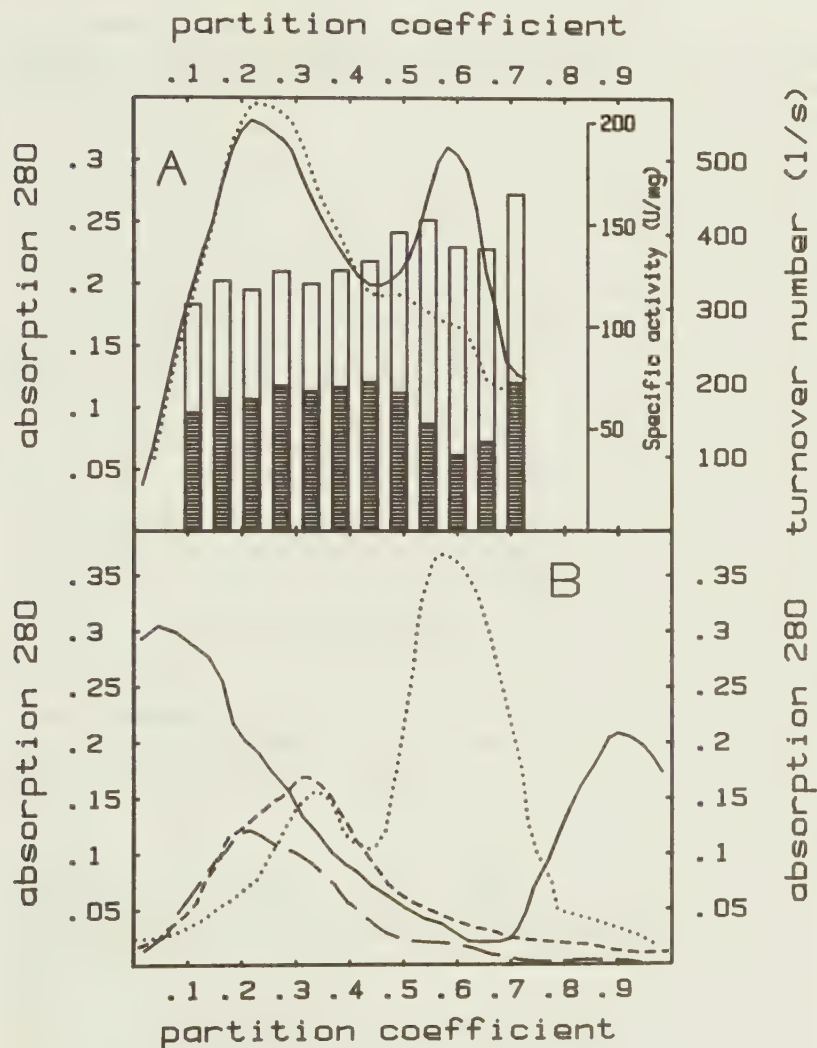


Figure 7: Elution profile of oxidase

Panel A : Elution of oxidase in 0.1% laurylmaltoside, 10 mM tris-HCl pH 7.8, and 100 mM KCl on a S-300 column (2.4 * 80 cm). (—) absorption at 280 nm, (·····) absorption at 420 nm (left ordinate divided by 2.5), open bars : turnover number (s^{-1}), hatched bars : specific activity (U/mg).

Panel B : (—) Elution of oxidase in 0.2% Tween-80, 10 mM tris-HCl pH 7.8, and 100 mM KCl on a S-300 column (0.9 * 60 cm). (---) pool #3 rechromatographed on a S-300 column (0.9 * 60 cm) (-----) pool #6 rechromatographed, (·····) pool #9 rechromatographed.

pools #1 to #8. After 24 h at 4 °C the cells were scanned and the molecular weight was analyzed. The molecular weight was found to be slightly inhomogeneous in all fractions. After 48 h it became even more inhomogeneous, so that no exact molecular weight determination was possible.

fraction (no.)	hydrodynamic radius	approx. molecular mass (kDa) *
1	800 - 1000	221 / 580
2	650 - 750	371 / 627
3	480 - 580	198 / 633
4	360 - 440	384 / 613
5	280 - 340	227 / 415
6	210 - 250	246 / 410
7	160 - 190	278 / 431
8	120 - 150	195 / 336

* The numbers refer to the two slopes obtained by plotting the sedimentation data according to equation 9.

Table 4: Comparison of molecular weight and hydrodynamic radius of oxidase

a) Detergent dependence of molecular weight

Laurylmaltoside as opposed to Tween-80, efficiently diminishes the amount of oligomeric oxidase. In Tween-80 about 50% of the oxidase elutes with an apparent molecular weight of 800'000 or higher, whereas in laurylmaltoside the fraction of oligomeric oxidase is always less than 5%.

b) Ionic strenght dependence of molecular weight

Elution of oxidase in 0.1% laurylmaltoside, 10 mM tris-HCl pH 7.4 without additional salt resulted in a larger amount of UV absorption eluting with an apparent molecular weight of 80'000 - 100'000 (Figure 8 A, dotted line). This size, however, corresponds to the size of the laurylmaltoside micelles. As gel filtration materials tend to behave as weak ion exchangers at very low ionic strength, some of the oxidase sticks to the column matrix. This oxidase is eluted together with the micelles. The subunit composition of oxidase eluting with an apparent molecular weight of 80'000 - 100'000 is the same as that of native oxidase. Hence, summing up the molecular weights of the subunits results in a molecular weight much larger than expected from the elution position in low ionic strength buffer. The monomer reported by Bolli et al. (1985) seems to be such an artifact of gel filtration.

c) Concentration dependence of molecular weight

Gel filtration of oxidase at 4 different concentrations was performed (45, 9, 5.5, 2.7 μM initial concentration). No significant difference in the monomer / dimer ratio was detected.

3.1.2 Conclusion

The elution profile of oxidase from different gel filtration columns in buffers with different detergents, or of different ionic strength is rather variable. The different aggregation state of oxidase in different detergents may be a first reason to explain this observation. Second, different amounts of detergent are bound to the oxidase in buffers of different ionic strengths. Third, the ion exchanging capability of gel filtration columns at low ionic strength may retard elution. In conclusion, it is not possible to correlate the partition coefficient with the molecular weight. Furthermore, the different oxidase species are in slow equilibrium with each other. Aggregation of monomers to a mixture of dimers and monomers takes roughly 24 hours. This is visible in a sedimentation equilibrium run on an analytical ultracentrifuge. Sedimentation studies with 'monomer' fractions from the S-300 column showed increasing amounts of dimers when they were repetitively scanned at 5 hour intervals.

Two explanations for the inability of gel filtration to separate solubilized monomers from dimers seem possible. First, the monomers and dimers are in equilibrium on the time scale of a gel filtration experiment (12-24 hours), but not during a short sedimentation velocity run (1 hour). A second and probably more satisfactory explanation is that self-association of the cytochrome c oxidase complex does not greatly increase the R_s of the complex. The R_s of two identical touching spherical balls is only 30% larger than a simple sphere. In the case of oxidase dimerization, R_s should increase less than 30% since (i) oxidase is known to be asymmetric and to self associate side by side and (ii) dimerization of a detergent solubilized protein almost certainly displaces some bound detergent from the hydrophobic protein surfaces in contact with each other. A simple geometric model based on the known molecular dimensions of oxidase ($115 \times 50 \times 50 \text{ \AA}$) and assuming a 35-40 $\text{\AA}$ layer of bound detergent results in a mere 18% increase of R_s in the dimer compared to the monomer (Robinson and Talbert, 1986).

In no case it was possible to elute a homogeneous species. Therefore, gel filtration had to be abandoned as a tool for the separation of oxidase into monomers and dimers.

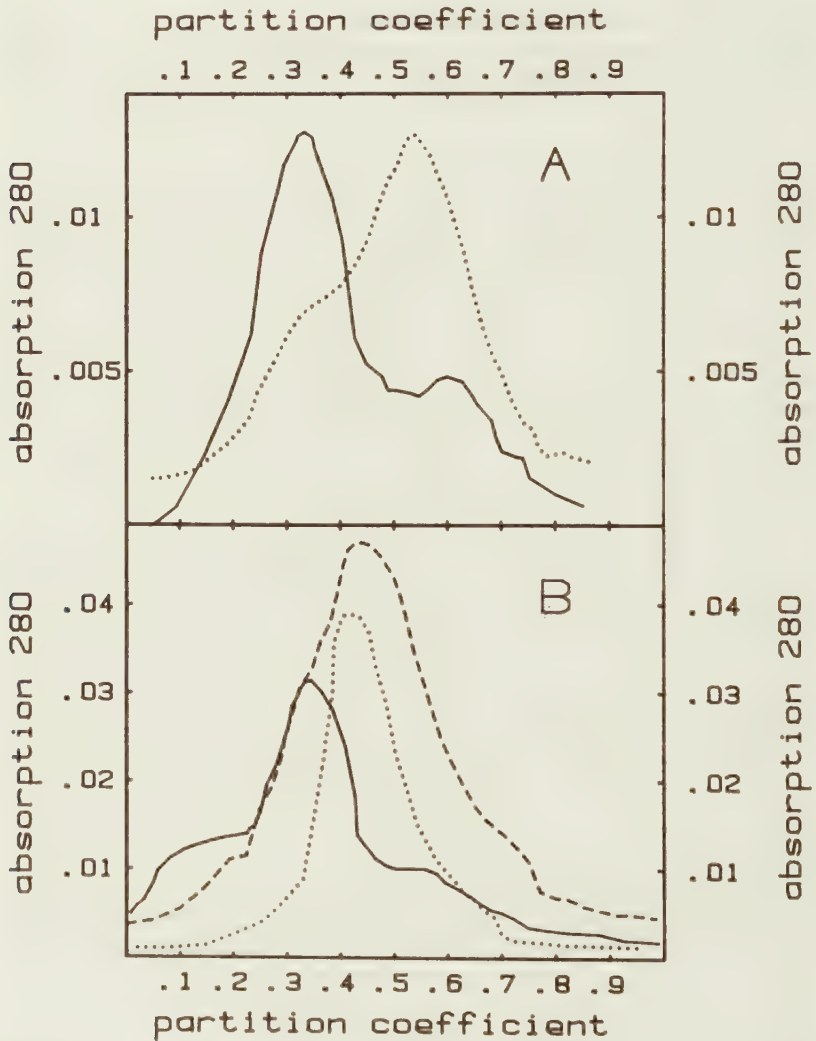


Figure 8: Monomeric oxidase

Panel A : Separation of oxidase in 10 mM tris-HCl pH 7.8, 0.1% laurylmaltoside on a S-300 column (0.9 * 60 cm), without salt (······) and with 100 mM KCl (———).

Panel B : (———) Elution of high pH treated oxidase on a S-300 column (0.9 * 60 cm) in 10 mM tris-HCl pH 7.4, 0.1% laurylmaltoside, and 100 mM KCl. (-----) elution of high pH and Triton X-100 treated oxidase. (······) rechromatography of same oxidase in same buffer.

3.1.3 Alkali and Triton X-100 Treated Oxidase

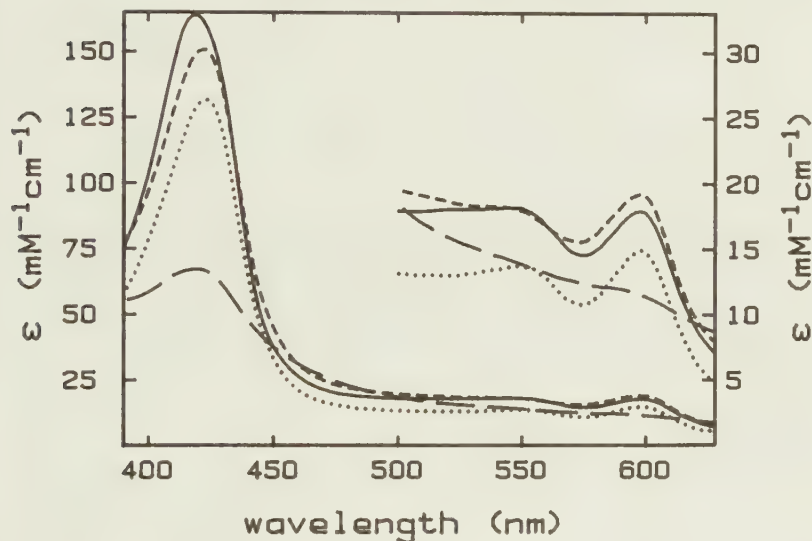


Figure 9: Spectra of monomeric oxidases

Spectrum of monomeric beef heart oxidase (—), Spectrum of *P. denitrificans* oxidase (·····), Spectrum of chymotrypsin treated beef heart oxidase (-----), oxidase subcomplex I-III (— · —).

Cytochrome c oxidase was treated with 5% Triton X-100 in 320 mM tris-HCl pH 8.5 as described in Methods. The enzyme was then applied to a DE-32 column, eluted with 10 mM tris-HCl, 200 mM NaCl, and 0.1% laurylmaltoside and concentrated by ultrafiltration in 10 mM tris-HCl pH 7.4, 15 mM NaCl, and 0.1% laurylmaltoside. Chromatography on a S-300 column (0.9 * 60 cm) resulted in a symmetrical peak eluting with an apparent molecular weight of 250'000 (Figure 8 B, dotted line). The stability of this oxidase was tested by rechromatography in low and high ionic strength buffers.

Sedimentation equilibrium studies were carried out at the same ionic strength and in the same detergent as was used for determining the molecular weight by gel filtration. The equilibrium distribution of oxidase was recorded in water and D₂O. Plots of the logarithm of concentration against the square of radial displacement ($\ln c$ vs. r^2) were slightly nonlinear (after 14h and 38h). The nonlinearity was found to be due to impurities incorporated in laurylmaltoside micelles. These impurities are detected by the optical system of the analytical ultracentrifuge ($\lambda = 280$ nm). Despite of the nonlinearity the slopes at the

two solvent densities (2 determinations each) could be determined. Extrapolation of the apparent molecular weight to the density of laurylmaltoside ($1/v_d = \rho_d = 1.22 \cdot 10^{-3} \text{ kg}^1 \text{m}^{-3}$; Suarez et. al., 1984) and correction for exchanged protons resulted in effective molecular masses of protein and detergent as shown in the Table below:

Protein	Micelle	Complex
200+-20 kDa	117 +-10 kDa	320+- 20 kDa

SDS-PAGE showed that oxidase treated with Triton X-100 at high pH and native oxidase had the same polypeptide composition. The Triton X-100 - high pH treated enzyme served as "monomeric" oxidase in binding and steady-state kinetic experiments. It was compared to the "dimeric" oxidase from the same preparation that had not been treated with Triton X-100 at high pH.

3.2 Spectroscopic Titration of Oxidase with Cytochrome c

Prior to the spectroscopic titrations the stability of the photometer, the mixing efficiency, and the reproducibility of the titrations were tested. The results of the test are listed in Part Two of this volume.

3.2.1 Optical Stability of Enzyme Solutions

The absorption, CD and MCD signal of 10 μM ferri- and ferrocytochrome c solutions were measured at 25°C for 1 hour periods. The stability of these solutions was always better than the stability of the photometer. Constant irradiation of the sample in the HP 8451 diode array photometer resulted in severe bleaching of the chromophore. This could be prevented by cutting off the short wavelengths (lower than 250 nm) with a glass cuvette or a filter.

Before the spectroscopic titrations of the oxidase with cytochrome c the optical stability of the oxidase was determined. A solution of oxidase (5 μM) was constantly stirred with a magnetic stirrer, 40 times 10 μl of buffer were added, the absorption was measured and the dilution was corrected for. The absorption spectrum was not very stable, the absorption at 420 nm increased or decreased in a random manner. Repetition of the stability measurements resulted in a near zero average. The maximal instability during a single titration was -0.002 ± 0.003 AU.

CD and MCD spectra of oxidase remained stable for 3 hours at 25 °C, much longer than the duration of normal measurements.

3.2.2 Spectroscopic Titration of Dimeric Oxidase

Spectroscopic titrations of oxidase in Tween-80 were performed in order to proof that the mathematical stray light correction procedure was equivalent to the physical stray light correction that was used in Michel (1984). The mathematical stray light correction has the advantage that no baseline has to be subtracted from the titration curve. As this method needs only a single cuvette, the noise is smaller than with the previous method. In addition to that, the procedure can be easily automated (see Part Two).

Laurylmaltoside is certainly the detergent of choice for measurements of physical properties of solubilized oxidase (VanAken, 1986). It is most effective in dispersing oxidase oligomers. Moreover, cytochrome c oxidase shows the best reaction rates in laurylmaltoside (Rosevear, 1980). The use of this detergent in all experiments of this study was prevented by the high costs of commercial laurylmaltoside. In addition to that, the synthetic procedure needed quite expensive starting materials and the purification was rather time consuming.

a) Spectroscopic titration in Tween-80

The cytochrome c oxidase prepared according to Hartzell and Beinert was dialyzed against 10 mM tris-HCl pH 7.4, 15 mM NaCl, and 0.2% Tween-80. The absorption

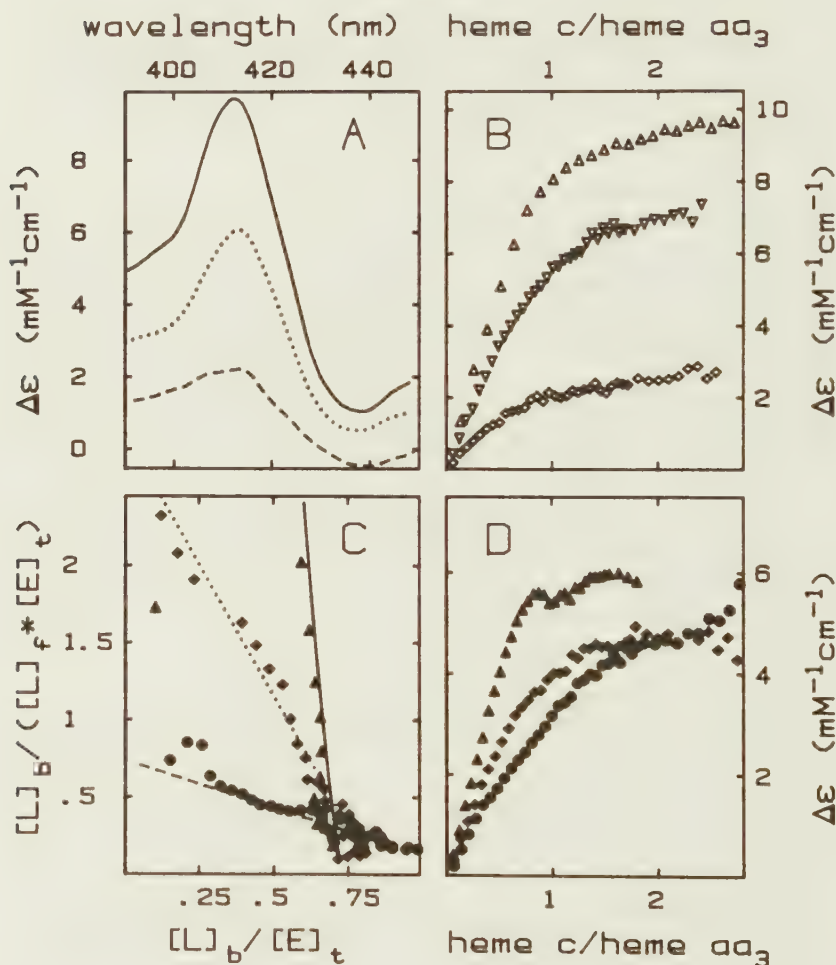


Figure 10: Spectroscopic titration of dimeric beef heart oxidase

Panel A : Difference spectra at saturation due to complex formation in Tween-80 : Ionic strengths : (—) $\mu=24$ mM, (.....) $\mu=49$ mM, (-----) $\mu=99$ mM.

Panel B : Titration curves of absorption difference at 414 nm in Tween-80 : (Δ) $\mu=24$ mM, (∇) $\mu=49$ mM, ($\diamond$) $\mu=99$ mM.

Panel C : Eadie Scatchard transformation of panel D with fitted curves (see text). (Δ , —) 24 mM, ($\diamond$,) 49 mM, ($\bullet$, -----) 74 mM.

Panel D : Titration curves of absorption difference at 414 nm in laurylmaltoside : Ionic strengths : (Δ) $\mu=24$ mM, ($\diamond$) $\mu=49$ mM, ($\bullet$) $\mu=74$ mM.

spectrum of a 1:1 complex of fully oxidized oxidase and ferricytochrome c in Tween-80 was significantly different from the absorption spectra of the components. The difference spectrum (spectrum of the complex minus summed spectra of components, Figure 10 A) had a maximum at 412 to 414 nm. The amount of bound cytochrome c could be titrated using the absorption difference at 414 nm. Extrapolation to infinite cytochrome c concentration resulted in a difference extinction coefficients of $\Delta\epsilon=10.1 \text{ mM}^{-1}\text{cm}^{-1}$ at 24 mM ionic strength, $7.7 \text{ mM}^{-1}\text{cm}^{-1}$ at 49 mM ionic strength, and $3.2 \text{ mM}^{-1}\text{cm}^{-1}$ at 99 mM ionic strength. The ionic strengths were achieved by addition of 24 mM and 74 mM NaCl, respectively.

b) Spectroscopic titration in laurylmaltoside

Cytochrome c oxidase was equilibrated into laurylmaltoside by gel filtration on an AcA-54 column. It was titrated with cytochrome c as described in Methods. The difference spectrum (spectrum of the complex minus summed spectra of components) had a maximum at 412 to 414 nm. This was exactly the same wavelength as in Tween-80. The amount of bound cytochrome c could be titrated using the absorption difference at 414 nm. Extrapolation to infinite cytochrome c concentration resulted in maximal extinction coefficients of $6.2 \text{ mM}^{-1}\text{cm}^{-1}$, $5.1 \text{ mM}^{-1}\text{cm}^{-1}$, and $6.6 \text{ mM}^{-1}\text{cm}^{-1}$ at 24 mM, 49 mM and 99 mM ionic strength, respectively.

3.2.3 2-Site Models versus 1-Site Model

Although graphical analysis is a quick and useful way to visualize binding data, for any serious study the data must be subjected to statistical analysis, in order that the quality of the fit can be quantitatively evaluated. This is particularly important if one wants to distinguish among different possible models (Klotz and Huston, 1978; Feldmann, 1972).

One of the most difficult problems was the graphical evaluation of the maximal difference signal. In Michel (1984) this maximal difference was determined by extrapolation from a reciprocal plot. Scatchard plots calculated using the graphically determined maximal absorption difference were non-linear, indicating negative cooperativity. A computer program was used to calculate the sum of squares of differences between experimental and calculated difference signals. Starting from estimates taken from the slope of the Scatchard graph the parameters were optimized by visual adaptation to the data points. We arrived at a negative cooperativity model on the basis of a graphical procedure. The result was interpreted to indicate negative cooperativity between the monomers of dimeric oxidase.

In order to be able to perform a statistical analysis several sets of data have to be measured under identical conditions. This led to the development of an automatic data acquisition system (see Part Two).

The most commonly used quantity for the quality of a fit is the least square. This is the sum of squares of differences between experimental and calculated signals when the parameters of a model are optimal. For the determination of least squares a non-linear regression program was developed (see Part Two). This program uses a weighted least

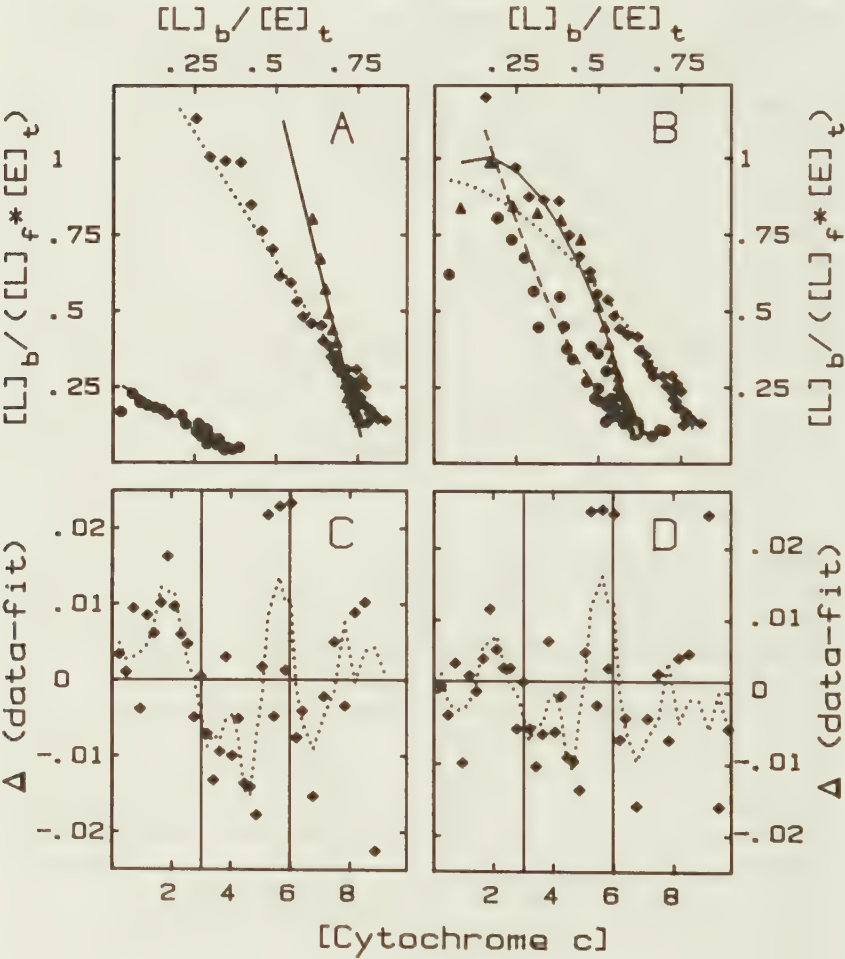


Figure 11: Discrimination among binding models

Panel A and B: Eadie Scatchard transformation of binding data together with simulated curves from the result of the fits (see text). Panel A : 1-site model, Panel B : 2-site model.
Panel C and D Residual plots between data and simulated curves of Panel A and B. Dotted lines : moving average of 3 consecutive points.

squares gradient search routine. The weight of each data point (ω_i in equation 12) was calculated from the inverse variance of the experimentally determined signal of several independent measurements.

If the same theoretical model is fitted to different sets of data the least square is an excellent means to select the best set of data. Much more complicated is the relation of the actual quality of the fit to the least square if different models are used on the same set of data (Cornish-Bowden and Endrenyi, 1986). In classical regression analysis the number of degrees of freedom is simply the difference between the number of observations and the number of parameters. However, in weighted least square fits some observations are weighed much more than others. Therefore, a classical calculation applied to the results of a weighted regression usually exaggerates the number of freedoms. The effective number of degrees of freedom (F_{eff}) is calculated as the effective number of observations (n_{eff}) minus the number of parameters.

$$n_{\text{eff}} = n - \frac{\sqrt{\sum (\omega_i - \bar{\omega})^2}}{2 * \bar{\omega}} \quad (12)$$

$$F_{\text{eff}} = n_{\text{eff}} - \text{number of parameters} \quad (13)$$

Models with different effective degrees of freedom are no longer directly comparable by their least squares. There is no general rule for the conversion of these different least squares into a comparable form. From experience with a large number of fits it can be deduced that division of the least squares by the number of degrees of freedom yields a very good quantity for the comparison of models. The comparison among least squares of different models is more reliable when a large number of data points have been measured.

Direct curve fits to the titration curves were performed using a 1-site model and several 2-site models.

$$\text{1 binding site : } r = \frac{[L]_b}{[E]_t} = \frac{K * [L]}{1 + K * [L]} \quad (14)$$

$$\text{2 binding sites: } r = \frac{[L]_b}{[E]_t} = \frac{K_1 * [L] + 2 * K_1 * K_2 * [L]^2}{1 + K_1 * [L] + K_1 * K_2 * [L]^2} \quad (15)$$

Where $[L]_b$ is bound ligand, $[L]$ free ligand, $[E]_t$ the enzyme concentration, r the fraction of enzyme with bound ligand, and K, K_1, K_2 the stoichiometric site binding constants (Klotz and Huston, 1978). It should be noted that $K_2 = 4 K_1$ for a 2-site model with equal independent binding sites. $K_2 < 4 K_1$ indicates positive cooperativity while $K_2 > 4 K_1$ is either due to negative cooperativity or to two a priori different binding sites. In addition, direct curve fits have been performed with a model based on microscopic site binding constants

a) fits to spectroscopic titrations in Tween-80

	μ (mM)	K_{d1} (μ M)	K_{d2} (μ M)	$\Delta\epsilon_{\text{max}}$ $\text{mM}^{-1}\cdot\text{cm}^{-1}$	noise *
1-site binding model	24	0.23	----	10.2	0.13
	49	0.59	----	7.9	0.10
	99	1.59	----	3.15	0.09
2-site binding model	24	0.33	0.37	10.1	0.13
	49	0.45	0.88	7.7	0.10
	99	0.26	4.9	3.44	0.09

b) fits to spectroscopic titrations in laurylmaltoside

	μ (mM)	K_{d1} (μ M)	K_{d2} (μ M)	$\Delta\epsilon_{\text{max}}$ $\text{mM}^{-1}\cdot\text{cm}^{-1}$	noise *
1-site binding model	24	0.07	----	6.15	0.12
	49	0.29	----	5.03	0.10
	74	1.63	----	6.57	0.12
2-site binding model	24	0.03	0.15	5.85	0.098
	49	0.31	0.77	5.14	0.11
	74	2.31	3.88	6.85	0.14

* root of least squares

Table 5: Comparison among binding models

and one with a priori identical but cooperative sites (Gormann and Darnall, 1981; José, 1985). The least squares that have been obtained for equation 15 were indistinguishable from the least squares of other 2-site binding models.

The effective degree of freedom for a weighted least square fit of the 1- and 2-site models (equations 14 and 15) have been calculated with equations 12 and 13. The 1-site binding model had an effective number of freedom of 35, and the 2-site binding model one of 34.

Is always the model with the smallest relative mean root square the best model ?

This is only true when all the models have the same degree of freedom (the same number of variables). The decision among models with different degrees of freedom is more com-

plicated. In this case the models have to be compared by their normalized least square. If models with lower degrees of freedom have a comparable relative mean root square, they have to be rejected. But if the relative mean root square is considerably smaller - at least 30% in my experience - a model has to be tested more thoroughly. One of the most important cues for the selection of a model is the difference between the actual data and the curve generated for the best fit as a function of ligand concentration. The differences should show a random distribution around zero, there must not be a preference for positive or negative sign in any part of the curve. (Figures 11 B and C).

In conclusion, spectroscopic titrations clearly result in one cytochrome c binding site per oxidase monomer, that is per heme aa₃. On the basis of the data presently available a two site binding model can be excluded for the binding of cytochrome c to monomeric, dimeric beef heart and *P. denitrificans* oxidase.

3.2.4 Spectroscopic Titration of Monomeric Oxidases

a) Beef heart oxidase

Beef heart oxidase monomers were prepared as described in methods, they were equilibrated into laurylmaltoside using a DE-32 ion exchange column (1.5 * 8 cm). They were titrated in 10 mM tris-HCl pH 7.4 and 0.1% laurylmaltoside with 15 mM and 40 mM NaCl. The difference spectra due to binding of cytochrome c (Figure 12 A) were maximal at 412 to 414 nm. This was exactly the same wavelength as with the dimeric oxidase. The amount of bound cytochrome c could be titrated using the absorption difference at 414 nm. Extrapolation to infinite cytochrome c concentration resulted in maximal difference extinction coefficients $\Delta\epsilon_{\max}$ of 6.2 and 8.1 mM⁻¹cm⁻¹ at 24 mM and 49 mM ionic strength, respectively.

b) *P.denitrificans* oxidase

P.denitrificans oxidase was a kind gift from B. Ludwig, Luebeck. It was diluted into 10 mM tris-HCl pH 7.4, 15 mM NaCl and 0.1% laurylmaltoside. The absorption spectrum of a 1:1 complex of fully oxidized *P.denitrificans* oxidase and ferricytochrome c in laurylmaltoside was significantly different from the absorption spectra of the components. The difference spectrum (Figure 13 A) had a maximum at 406 nm. This was a 6 nm blue shift compared to the difference spectrum of beef heart oxidase. The amount of bound cytochrome c could be titrated using the absorption difference at 406 nm. Extrapolation to infinite cytochrome c concentration resulted in maximal difference extinction coefficients $\Delta\epsilon_{\max}$ of 6.8 and 3.4 mM⁻¹cm⁻¹ at 24 mM and 49 mM ionic strength, respectively.

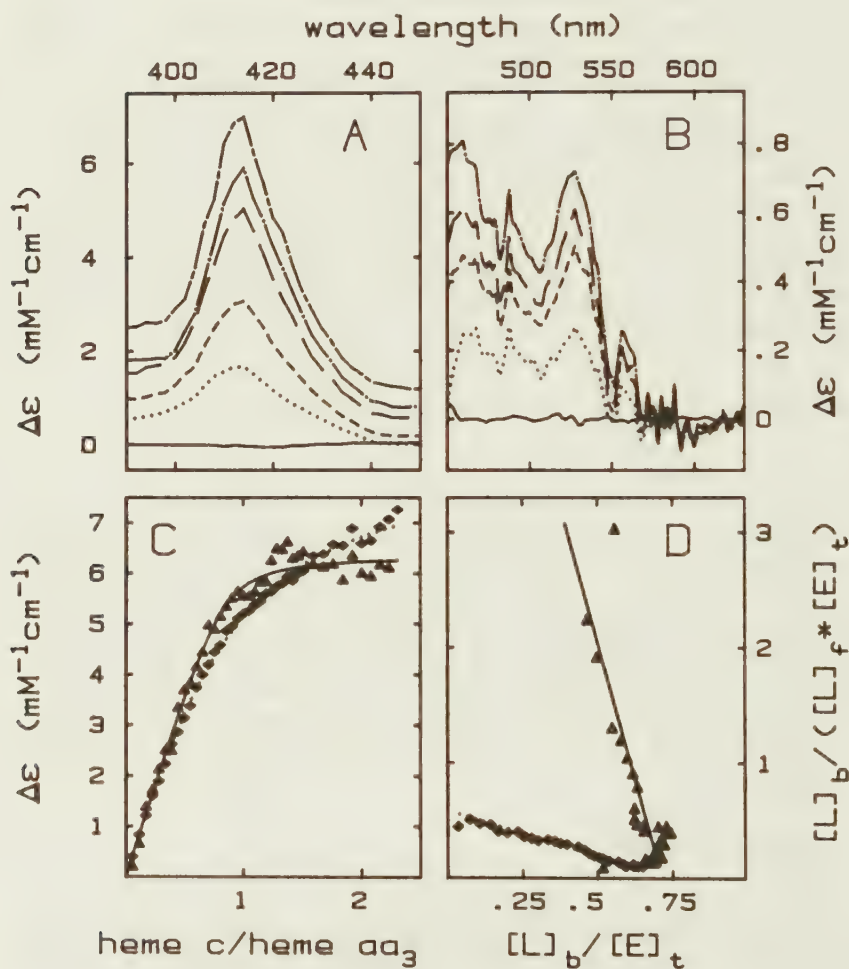


Figure 12: Spectroscopic titration of monomeric oxidase

Panel A : Difference spectra at 49 mM ionic strength with variable ratios of heme c to heme aa₃. (—) 0, (·····) 0.25, (-----) 0.5, (— — —) 0.75, (— · — · —) 1, and (— — —) 2.

Panel B : Difference spectra from 450 to 600 nm (see panel A).

Panel C : Spectroscopic titration of monomeric oxidase at 414 nm, (▲) 24 mM ionic strength, (♦) 49 mM ionic strength. The lines are drawn for the same fits as in panel D.

Panel D : Scatchard transformation of titration curves with fits for 1-site model :

(▲, ———)	: $K_D = 0.11$ (μM)	$\Delta\epsilon = 6.2$ $\text{mM}^{-1}\text{cm}^{-1}$	root least square = 0.15
(♦, ·····)	: $K_D = 1.27$ (μM)	$\Delta\epsilon = 8.1$ $\text{mM}^{-1}\text{cm}^{-1}$	root least square = 0.09

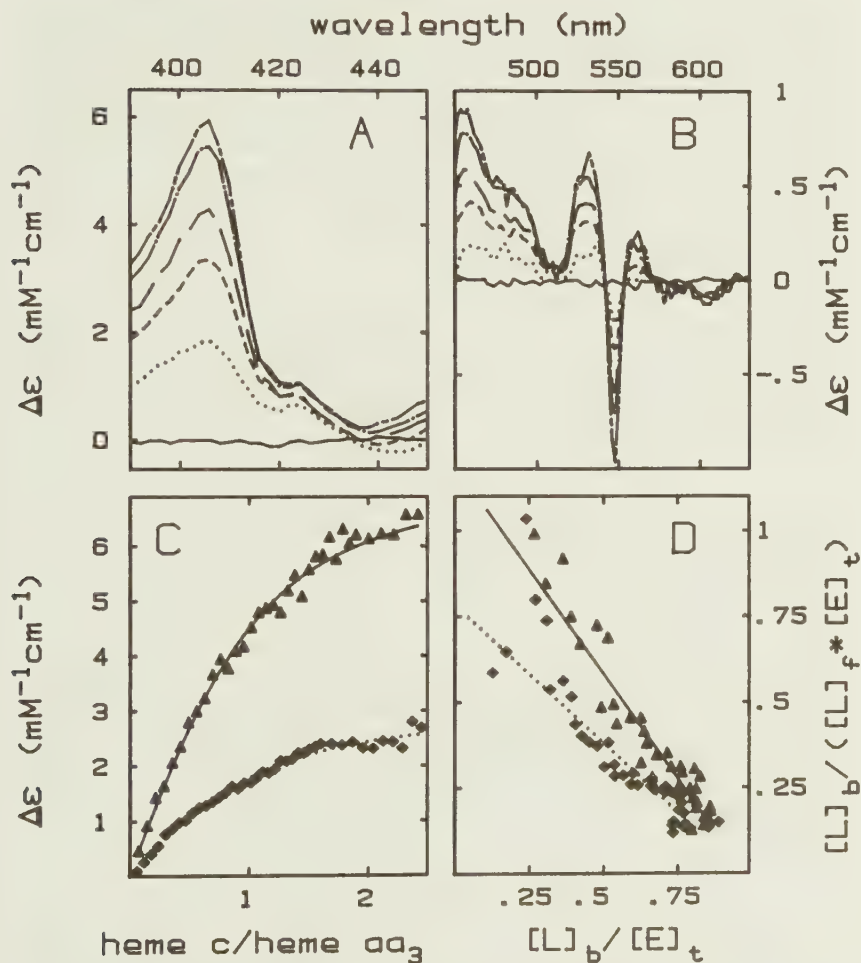


Figure 13: Spectroscopic titration of *P. denitrificans* oxidase

Panel A: Difference spectra at 49 mM ionic strength with variable ratios of heme c to heme aa_3 .

(—) 0, (·····) 0.25, (-----) 0.5, (— · — ·) 0.75, (— · — —) 1, and (— — —) 2.

Panel B: Difference spectra from 450 to 600 nm (see panel A).

Panel C: Spectroscopic titration at 406 nm, ($\blacktriangle$) 24 mM ionic strength, ($\blacklozenge$) 49 mM ionic strength. The lines are drawn for the same fits as in panel D.

Panel D: Scatchard transformation of titration curves with fits for 1-site model:

($\blacktriangle$, —) : $K_D = 0.86$ (μM) $\Delta\epsilon = 6.77 \text{ mM}^{-1}\text{cm}^{-1}$ root least square = 0.18
 ($\blacklozenge$, ·····) : $K_D = 0.97$ (μM) $\Delta\epsilon = 3.35 \text{ mM}^{-1}\text{cm}^{-1}$ root least square = 0.10

3.3 Steady-State Oxidation of Cytochrome c by Oxidase

Steady-state oxidation of ferrocycytochrome c by oxidase was determined at 20 different concentrations by means of the 'ASSAYOMATE' and the 'MICHKIN' software described in Part Two. The oxidation of ferrocycytochrome c was strictly first order under all conditions of pH, ionic strength, detergent, and temperature. The different substrate concentrations were mixed from the same stock solution using the "ASSAYOMATE". Kinetic models were fitted to the experimental data with the "MICHFIT" software on a HP 150 personal computer.

3.3.1 Models for Oxidase Kinetics

a) Existing models

A large number of models have been proposed for cytochrome c oxidase kinetics (see Introduction). At high ionic strength and at low pH, the oxidase behaves like a Michaelis Menten enzyme :

$$\frac{1}{[E]_0} * \frac{d[S]}{dt} = \frac{TN_{max} * [S]}{K_m + [S]} \quad (17)$$

At low ionic strength and neutral to alkaline pH the kinetics show non-linear Eadie - Hofstee plots which, in the simplest case, can be described by :

$$\frac{1}{[E]_0} * \frac{d[S]}{dt} = \frac{TN_{max1} * [S]}{K_{m1} + [S]} + \frac{TN_{max2} * [S]}{K_{m2} + [S]} \quad (18)$$

Equation 18 was selected to fit the non-linear Eadie Hofstee plots because the fitted parameters have a simple meaning. Formally, the equation describes two independent enzymes acting on the same substrate. As the oxidase is a proton-pumping enzyme it must have two conformations with different properties which can be regarded as two independent enzymes acting on the same substrate. These conformers occur in different ratios depending on conditions (pH, ionic strength). The two TN_{max} are thus proportional to the relative concentration of these two conformers and the two K_m are related to their respective affinity. Equation 18 can be converted to the following form :

$$\frac{1}{[E]_0} * \frac{d[S]}{dt} = \frac{A * [S] + B * [S]^2}{1 + C * [S] + D * [S]^2} \quad (19)$$

Parameters A and C describe the linear dependence of the velocity on the substrate concentration and parameters B and D describe the quadratic dependence. A to D are related to K_m and TN_{max} as follows :

$$A = (TN_{max1} * K_{m2} + TN_{max2} * K_{m1}) / (K_{m1} * K_{m2})$$

$$B = (TN_{max1} + TN_{max2}) / (K_{m1} * K_{m2})$$

$$C = (K_{m1} + K_{m2}) / (K_{m1} * K_{m2})$$

$$D = 1 / (K_{m1} * K_{m2})$$

Almost all kinetic equations of non-Michaelian enzyme reactions can be converted to a form in which the velocity depends on either the square or the third power of the substrate concentration. It is thus impossible to select a model on the basis of steady-state kinetics alone. In other words, the interpretation of the K_m and TN_{max} value depends on the reaction mechanism. Nevertheless, it was decided to use equation 18 as the simplest possible equation to fit the velocity data.

b) Comparison among models

It is not possible to distinguish the model by equation 18 and 19 from the dependent site mechanism or the independent site mechanism (see page 13) on the basis of steady-state turnover data (Mannervik, 1982). The single catalytic site model of Speck et al. (1984) can be converted to an equation with third power dependence on substrate concentration (equation 3). Fits of data to equation 3 resulted in least squares comparable to those of fits to equation 2. As the fits to the equation 3 have a smaller effective degree of freedom, the least squares had to be slightly smaller than for fits to equation 2 (see Introduction and Section 3 of this chapter). Due to lack of computing power (8088 processor, 256 Kbyte memory, no mathematical coprocessor !) it was not possible to perform the large number of fits that would have been needed to distinguish among these models.

Several authors explain biphasic kinetics by assuming a high affinity and a low affinity binding site. The Michaelis constant, however, is a dynamic or pseudo equilibrium constant expressing the relationship between the actual steady-state concentrations rather than equilibrium concentrations. It is possible to replace K_m by K_d if the conversion of the enzyme-substrate complex to the enzyme-product complex (k_2) is much slower than the off rate of the substrate (k_{-1}).

Spectroscopic titrations of the oxidized resting enzyme yield the dissociation constants of the enzyme-product complex. However, it has been shown that the dissociation constant of reduced cytochrome c is virtually identical. This could be supported by spectroscopic titration of the fully reduced enzyme (Michel, 1984).

Replacing K_{m1} in equation 2 by K_d resulted in bad fits. Thus, in the oxidase, k_2 is approximately of the same order of magnitude as k_{-1} .

3.3.2 Steady-State Oxidation by Dimeric Oxidase

Steady state turnover of ferrocycytochrome c by dimeric oxidase was biphasic at low ionic strength in all detergents that have been tested.

The experimental data could be fitted to a model with two Michaelis constants and two maximal velocities (equation 18). The least squares were always very small under all conditions. The points in the residual plots were randomly distributed around zero (Figure 11). The results of the fits, the two Michaelis constants K_{m1} , K_{m2} and the two maximal turnover numbers TN_{max1} , TN_{max2} together with the least squares are compiled in Table 6.

TN_{max1} and K_{m1} are termed the high affinity site values and TN_{max2} and K_{m2} the low affinity site values. The column at the left is the ionic strength (μ) of the assay buffer.

	μ (mM)	TN_{max1} (s^{-1})	K_{m1} (μM)	TN_{max2} (s^{-1})	K_{m2} (μM)	Noise *
lauryl- maltoside	24	77	0.73	9.5	33	0.22
	49	49	0.54	29	5.74	0.56
	74	56	1.51	35	7.8	0.91
	99	47	1.6	36	18.6	0.80
	150	----	----	69	6.6	1.06
Tween-80	24	7.2	0.43	54	27	0.21
	49	17	0.71	40	14	0.42
	74	25	0.75	65	13	1.2
Triton X-100	24	1.7	0.22	2.9	4.2	0.086
	49	0.97	0.12	2.3	2.7	0.056
	74	1.0	0.32	2.1	2.2	0.054
	99	0.79	0.27	1.3	2.3	0.033
	150	----	----	1.75	0.96	0.027

--- No second phase

* root of least square

Table 6: Kinetic constants of dimeric oxidase

The ionic strength was adjusted by the addition of sodium chloride (15, 40, 65, 90, 140 mM) to 10 mM tris-HCl pH 7.4 and 0.1% laurylmaltoside, or 0.2% Tween-80, or 0.2% Triton X-100.

Figure 14 A shows the steady-state activity of dimeric oxidase in laurylmaltoside at different ionic strengths. The lines drawn through the data points are curves generated from the results of the fits. The results of these fits are compiled in the upper third of Table 6. Maximal turnover of the high affinity site decreases when the ionic strength is increased. The opposite effect is observed for maximum turnover of the low affinity site. The Michaelis constant of the high affinity site increases with the ionic strength, starting from 49 mM. An exception to this rule is the value at 24 mM ionic strength. Above approximately 100 mM ionic strength the high affinity site merges with the low affinity site; for these conditions simple Michaelis-Menten kinetics can be applied. The low affinity Michaelis constant decreases upon increasing ionic strength until it merges with the high affinity phase. After that point the tendency changes and the merged constants increase.

Figure 14 C shows the steady-state reaction of dimeric oxidase in Tween-80. The results of the fits to these data are compiled in the middle part of Table 6. Maximal turnover of the high affinity site increases with increasing ionic strength. This is remarkably different from the behavior of the high affinity maximal turnover in laurylmaltoside. Maximal turnover at the low affinity site is approximately constant and thus differs from the behavior in laurylmaltoside too. Both high and low affinity Michaelis constant show a similar behavior as in laurylmaltoside.

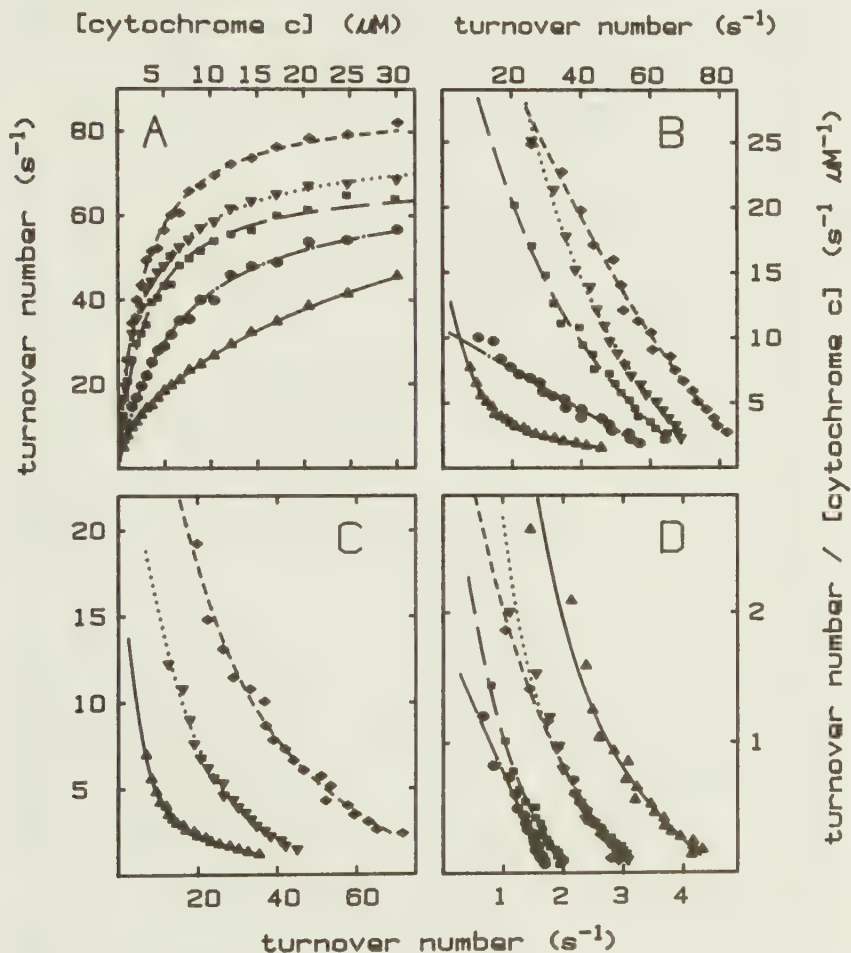


Figure 14: Steady-state oxidation by dimeric oxidase

The lines drawn through the data points are simulated curves generated with the result from fits as compiled in Table 6. Ionic strength : ($\blacktriangle$, —) 24 mM, ($\blacktriangledown$, ·····) 49 mM, ($\blacklozenge$, - - - -) 74 mM, ($\blacksquare$, — · — ·) 99 mM, and ($\bullet$, — · — ·) 150 mM.

Panel A : Steady-state activity of oxidase in laurylmaltoside.

Panel B : Eadie Hofstee transformation of data from panel A, same symbols.

Panel C : Eadie Hofstee transformation of steady-state activity of dimeric oxidase in Tween-80.

Panel D : Eadie Hofstee transformation of steady-state activity of dimeric oxidase in Triton X-100.

In Triton X-100 the maximal turnovers are strikingly low compared to those measured in laurylmaltoside and Tween-80. The Michaelis constants are different too but they show the same ionic strength dependence as in laurylmaltoside and Tween-80.

Wilms et al. (1981, 1982) have reported an optimal ionic strength of 75 mM for the steady-state activity of oxidase. We too observe the same optimal ionic strength for the sum of high and low affinity turnover numbers. The sums of $TN_{\max 1}$ and $TN_{\max 2}$ in laurylmaltoside are (s^{-1}) : 87, 89, 91, 75, and 69.

3.3.3 Steady-State Oxidation by Monomeric Oxidases

Steady-state turnover of ferrocycytochrome c by monomeric oxidases (beef heart and *Paracoccus denitrificans*) was biphasic at low ionic strength. The experimental data could be fitted to the same model (equation 2), with the same small least squares as in the case of dimeric oxidase.

Figure 15 A shows the steady state activity of monomeric beef heart oxidase in laurylmaltoside at different ionic strengths. The lines drawn through the data points are curves generated from the results of the fits. The results of these fits are compiled in the upper half of Table 7. Unlike with dimeric oxidase, the maximal velocity of the high affinity site increases when the ionic strength is raised, which is consistent with the result for dimeric oxidase in Tween-80. Maximal velocity of the low affinity site does not show a definite tendency. At high ionic strength, the two Michaelis constants of monomeric oxidase converge.

In Tween-80 all parameters of monomeric beef heart oxidase have the same ionic strength dependence and approximately the same magnitude (Figure 15 B, Table 7).

Figure 15 C shows the steady-state activity of *P. denitrificans* oxidase in laurylmaltoside at different ionic strengths. This oxidase shows a very pronounced ionic strength dependence. At low ionic strength (24 mM) two largely different Michaelis constants are observed ($K_{m1} = 0.2$, $K_{m2} = 15$). These two phases merge very quickly and at 74 mM ionic strength only a single phase is detected. The maximal velocity of the low affinity phase is low at 24 mM ionic strength, then increases up to 74 mM. At ionic strength higher than 74 mM this velocity starts to decrease. The low affinity phase Michaelis constant was more or less constant between 24 and 99 mM ionic strength.

The same picture results from the steady-state activity experiments at different ionic strengths in Tween-80. Again two Michaelis constants merge very rapidly when the ionic strength is raised from 24 mM to 74 mM. Unlike in laurylmaltoside the low affinity Michaelis constants are much higher especially at 24 mM (152 μM instead of 15 μM) and at 74 mM (48 μM instead of 14 μM).

The surprising but important picture that emerges from these experiments is that the inherent biphasic steady-state turnover of all oxidases does not depend on their aggregation state. As with dimeric oxidases the steady-state turnover of the monomeric oxidases is ionic strength dependent. They show a similar ionic strength optimum. The high affinity site Michaelis constant is approximately constant in different detergents, but the low

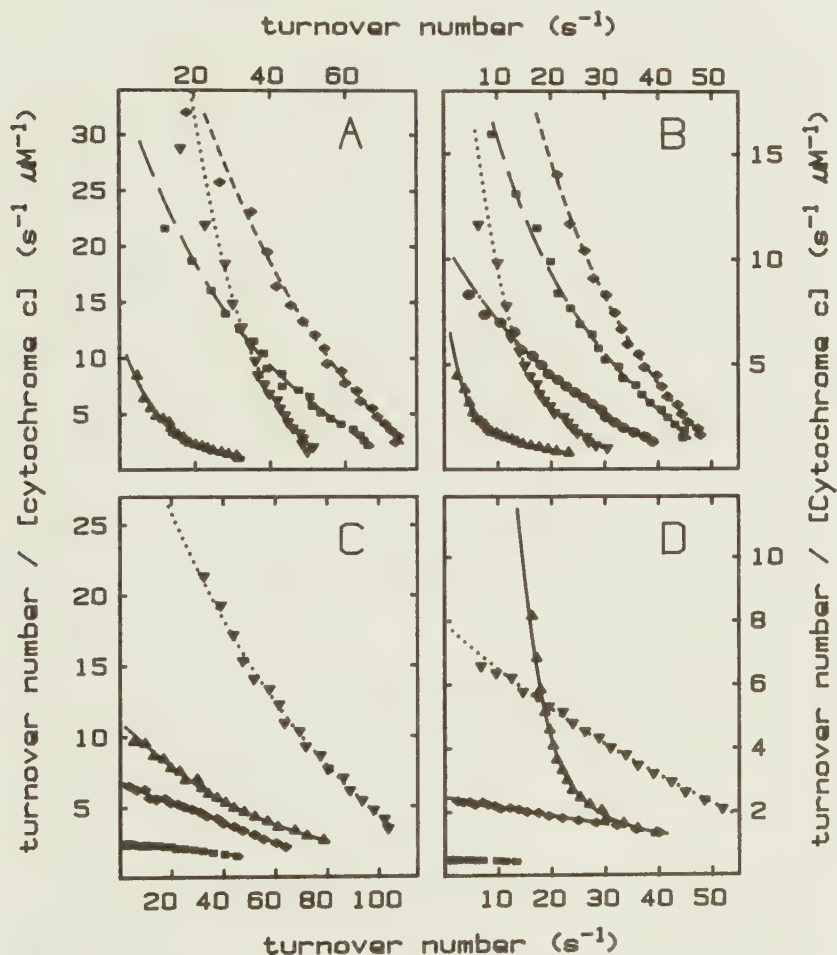


Figure 15: Steady-state oxidation by monomeric oxidases

The result of the fits are compiled in Table 7. Ionic strength: (▲, —) 24 mM, (▼, ·····) 49 mM, (◆, - - - -) 74 mM, (■, — · —) 99 mM, and (●, — · — · —) 150 mM.

Panel A: Eadie Hofstee transformation of steady-state activity of monomeric beef heart oxidase in 0.1% laurylmaltoside.

Panel B: Eadie Hofstee transformation of steady-state activity of monomeric beef heart oxidase in Tween-80.

Panel C: Eadie Hofstee transformation of steady-state activity of *P. denitrificans* oxidase in laurylmaltoside.

Panel D: As Panel C but in Tween-80.

a) monomeric beef heart oxidase

	μ (mM)	TN_{max1} (s ⁻¹)	K_{m1} (μ M)	TN_{max2} (s ⁻¹)	K_{m2} (μ M)	Noise *
lauryl-maltoside	24	39	1.0	10	21	0.37
	49	30	0.3	26	5.3	0.56
	74	44	0.8	39	5.6	0.70
	99	55	0.9	23	7	0.76
Tween-80	24	4	0.6	39	31	0.12
	49	11	0.4	26	12	0.31
	74	22	0.7	32	6.5	0.30
	99	13	0.7	39	6.3	0.43
	149	12	1.7	36	9.8	0.30

b) *P. denitrificans* oxidase

	μ (mM)	TN_{max1} (s ⁻¹)	K_{m1} (μ M)	TN_{max2} (s ⁻¹)	K_{m2} (μ M)	Noise *
lauryl-maltoside	24	34	0.2	92	15	1.17
	49	45	1.8	85	11	0.65
	74	0	---	92	14	0.32
	99	0	---	96	35	0.38
Tween-80	24	16.3	0.3	95	92	0.27
	49	22.8	4.3	59	20	0.26
	74	0	---	90	40	0.13
	99	0	---	80	150	0.06

--- no second phase * root of least square

Table 7: Kinetic constants of monomeric oxidases

affinity K_m is strongly dependent on the detergent. At 24 mM ionic strength it is 152 μ M in Tween-80 whereas in laurylmaltoside it is ten times lower.

The monomeric beef heart oxidase seems to be less stable in kinetic terms than the dimeric oxidase. Especially it is more subject to ageing in concentrated stock solutions as well as when diluted. But no instability of the absorbance was observed. The optical stability was at least as good as that of dimeric oxidase.

3.3.4 pH-Dependence of Steady-State Oxidation by Oxidase

	pH	$TN_{\max 1}$ (s^{-1})	K_{m1} (μM)	$TN_{\max 2}$ (s^{-1})	K_{m2} (μM)	Noise *
beef heart oxidase dimer	8.6	30	0.4	21	16	0.93
	8.0	59	0.5	52	2.4	2.4
	7.4	122	0.7	70	4.4	2.7
	6.8	265	1.6	0	---	3.3
	6.2	334	1.9	0	---	7.4
	5.6	344	2.4	0	---	3.4
beef heart oxidase monomer	8.6	7.4	0.6	22	9	0.2
	8.0	17	0.5	32	11	1.0
	7.4	77	2.0	64	67	1.1
	6.8	188	2.6	0	---	4.6
	6.2	259	3.1	0	---	5.4
	5.6	258	2.8	0	---	4.9
Paracoccus denitri- ficans	8.6	15	0.8	11	16	0.4
	8.0	35	1.7	44	16	1.3
	7.4	14	1.8	132	5.4	2.1
	6.8	187	2.6	---	---	3.5
	6.2	238	7.9	---	---	3.1
	5.6	270	9.9	---	---	2.0

* root of least square

Table 8: Kinetic constants at different pH-values

The steady-state oxidation of oxidase was measured at different pH-values at an ionic strength of 49 mM. Buffers with pH-values of 7.4, 8, and 8.6 were composed of 10 mM tris-HCl, 0.1% laurylmalto-side, and 47, 44, or 40 mM NaCl, respectively. Buffers with pH-values of 7.4, 6.8, 6.2, and 5.6 were composed of 10 mM bis-tris-HCl, 0.1% laurylmalto-side and 48, 47, 44.5, or 41 mM NaCl, respectively.

Steady-state turnover was biphasic at high pH values (pH 7.4, 8.0, and 8.6) and became monophasic when the pH was lowered (pH 6.8, 6.2, and 5.6). The experimental data could be fitted to equation 18 (above pH 7.4) and to equation 17 at pH 6.8 and lower. The results of these fits have been compiled in Table 8.

Figure 16 (Δ , solid lines) shows the steady-state activity of all oxidase species in laurylmalto-side at an ionic strength of 49 mM in 10 mM bis-tris-HCl pH 5.6. The Eadie Hof-stee transformations are perfectly linear. The maximal turnover of dimeric oxidase is 344 molecules cytochrome c per aag unit per second, a much higher value than at neutral pH (Figure 16 A, Δ , solid line). Monomeric beef heart oxidase is slightly less efficient but the Michaelis constant is identical (Figure 16 B, Δ , solid line). *P. denitrificans* oxidase attains almost the same maximal speed, but the Michaelis constant is four times higher (9.9 μM instead of 2.4 μM , Figure 16 C, Δ , solid line). A similar picture can be seen at

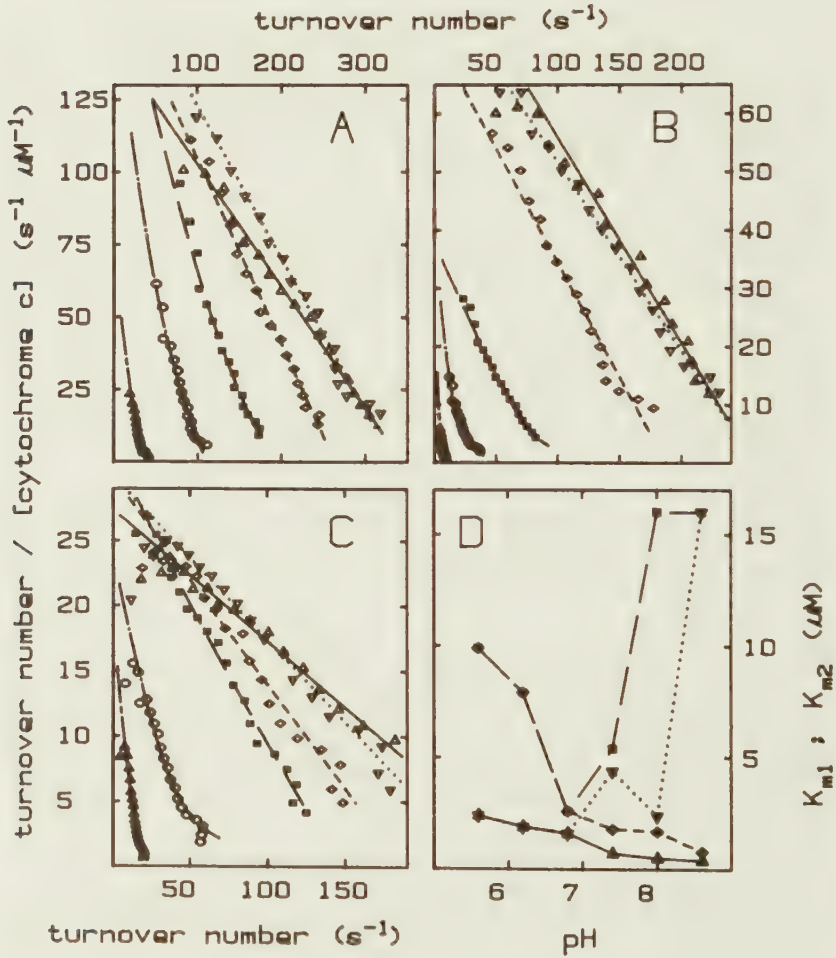


Figure 16: Steady-state oxidation at different pH
(Δ, —) pH 5.6, (▽, ·····) pH 6.2, (◊, -----) pH 6.8
(◻, — — —) pH 7.4, (○, — — — —) pH 8.0, (▲, — — — —) pH 8.6.
The lines drawn through the data points are simulated curves generated with the result from fits as compiled in Table 8.
Panel A : Eadie Hofstee transformation of steady-state activity of dimeric oxidase.
Panel B : Eadie Hofstee transformation of steady-state activity of monomeric beef heart oxidase.
Panel C : Eadie Hofstee transformation of steady-state activity of *P. denitrificans* oxidase.
Panel D : Plot of K_m as a function of pH. (—, K_{m1}; ·····, K_{m2}) of beef heart oxidase dimers from Panel A. (-----, K_{m1}; — — —, K_{m2}) of *P. denitrificans* oxidase from Panel C.

pH 6.2. Again, the maximal velocities and the Michaelis constants are higher than at neutral pH. When the pH is further increased, the Eadie Hofstee transformations become slightly non-linear (pH 6.8, Figure 16, $\diamond$, dashed lines). At neutral pH (pH 7.4, Figure 16, $\equiv$, long dashes) the Eadie Hofstee plots are definitely biphasic. At this pH the buffer medium had to be changed, but the steady-state activity of dimeric oxidase remained unchanged when measured either in tris or bis-tris buffers. The biphasicity becomes more pronounced when the pH is further increased.

Figure 16 A, B, and C, Δ shows the steady-state activity of all oxidase species at pH 8.6 (ionic strength 49 mM, 10 mM tris-HCl pH 8.6). The maximal turnover of dimeric oxidase is much lower than at neutral pH ($TN_{\max 1} + TN_{\max 2} = 51$). The high and low affinity Michaelis constants differ by a factor of 40 (0.4 μ M and 16 μ M, Δ , panel A). The same behavior is observed with monomeric beef heart and *P. denitrificans* oxidase.

An increasing pH results in a decreasing overall turnover. The single Michaelis constant is split into two Michaelis constants at pH-values above 6.8. This behavior is independent of the aggregation state or the origin of the oxidase.

The speed of the conformational change of the oxidase is dependent on the pH. At low pH this speed is larger than the cytochrome c binding rate. At higher pH this speed is lower, so that slow and fast turnover by the two different conformers is being observed. This tendency closely resembles the ionic strength dependence up to 74 mM.

3.3.5 Oxidase Subcomplexes

a) Chymotrypsin treated beef heart oxidase

Beef heart oxidase was treated with chymotrypsin as described in Methods. It was titrated with cytochrome c in 10 mM tris-HCl pH 7.4, 15 mM NaCl, and 0.1% laurylmaltoside. Binding of this oxidase to fully oxidized ferricytochrome c resulted in a spectrum that was different from the sum of the spectra of the components (Figure 17 A, solid line). The difference spectrum was maximal at 406 nm and minimal at 440 nm. The maximal difference of chymotrypsin treated oxidase is at exactly the same position as in the case of *P. denitrificans* oxidase ! The titration of the absorption difference at 406 nm revealed an inhomogeneous population of oxidase with an average affinity of 17 μ M.

Chymotrypsin treated oxidase was still capable of oxidizing ferrocycytochrome c, but at a much lower rate ($TN_{\max} = 2.73 \text{ s}^{-1}$). In contrast to all other oxidases the steady-state turnover catalyzed by chymotrypsin treated oxidase was strictly monophasic. The Michaelis constant was between the high and low affinity Michaelis constants of native oxidase (2.35 μ M). This monophasic turnover can be explained by a destroyed thermodynamical linkage of the electron transfer to the conformational change of the modified oxidase.

b) Resolution of beef heart oxidase into subunits

Beef heart oxidase was split into subunits as described in Methods. There was no detectable turnover of ferrocycytochrome c catalyzed by the subcomplex composed of subunits I - III. Mixing this subunit with ferricytochrome c resulted in a spectrum that was different

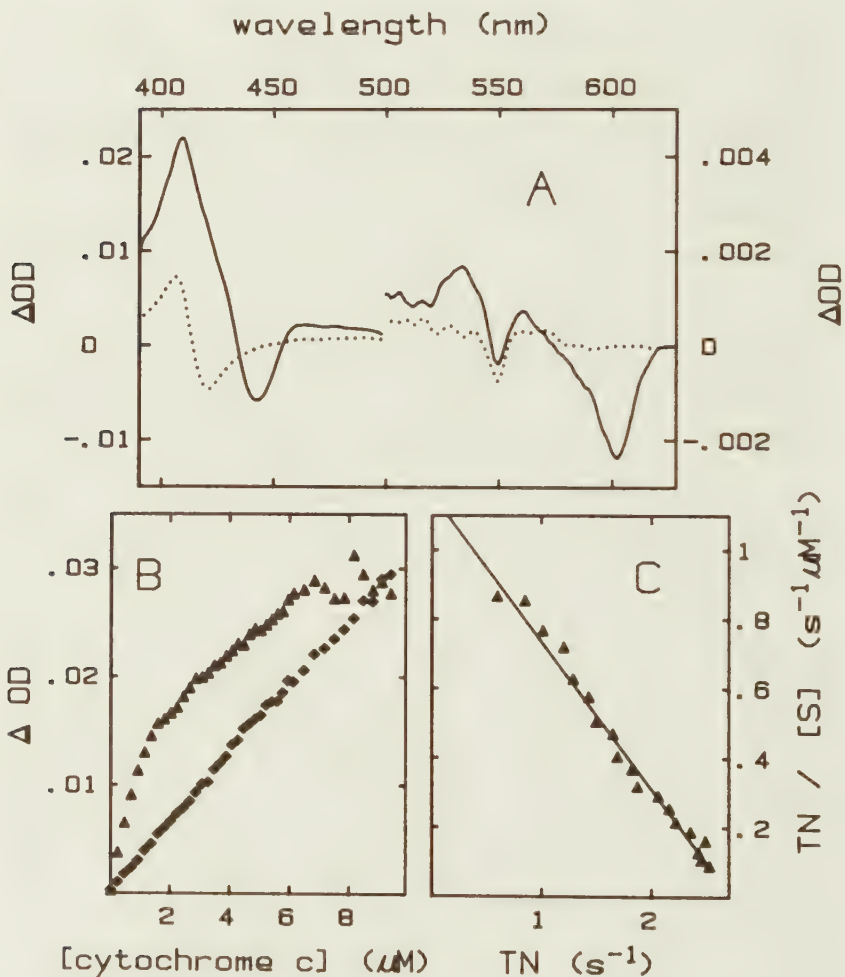


Figure 17: Oxidase subcomplexes

Panel A : (—) Absorption difference upon complex formation of 5 μM chymotrypsin treated oxidase with 5 μM ferricytochrome c. (.....) Absorption difference upon complex formation of a subcomplex of beef heart oxidase containing peptides I,II and III with 5 μM ferricytochrome c. Buffer : 10 mM tris-HCl pH 7.4, 15 mM NaCl, 0.1% laurylmaltoside.

Panel B : (Δ) Spectroscopic titration of chymotrypsin treated oxidase at 406 nm. ($\diamond$) Spectroscopic titration of the oxidase subcomplex (I,II, and III) at 404 nm.

Panel C : Eadie Hofstee transformation of steady-state activity by chymotrypsin treated beef heart oxidase. The line is drawn for the optimal fit : $K_M = 2.35 \mu M$ and $TN_{max} = 2.73 s^{-1}$.

from the sum of the spectra of the components (Figure 17 A, dotted line). The difference spectrum was maximal at 408 nm, a minimum was observed at 420 nm.

3.3.6 Summary of Binding and Kinetic Results

Binding of cytochrome c to beef heart oxidase causes a difference spectrum with a maximum at 414 nm (Figure 18 A, solid line). The extinction coefficient of the difference spectrum is $5.0 \pm 0.5 \text{ mM}^{-1}\text{cm}^{-1}$ at 49 mM ionic strength. This value is not strongly dependent on the aggregation state of the oxidase. In Tween-80 the differences at low ionic strengths are larger than in laurylmaltoside, but they are more affected by an increase in ionic strength. At lower ionic strength the signal is larger and at higher ionic strength it is smaller. Binding of ferricytochrome c to *P. denitrificans* oxidase causes a difference spectrum of approximately the same magnitude with a maximum at 408 nm. All difference spectra can be used to monitor the amount of bound cytochrome c. The titration curves saturate at a ratio of 1 cytochrome c bound per heme aag (with beef heart oxidase the amount of bound cytochrome c is about 0.85, this might be due to an inaccurate extinction coefficient for the oxidase). Thorough analysis of the binding data lead to exclusion of negative cooperativity between dimers. Moreover, negative cooperativity between adjacent binding sites of which only one site gives rise to a difference absorption signal can be excluded (Figure 18 B).

Steady-state kinetics at low ionic strength were non-linear in Eadie Hofstee plots; whereas at higher ionic strength the two phases merged to one single phase (Figure 18 E). Steady-state kinetics in buffers of different pH-value and 49 mM ionic strength showed a similar behavior of the two kinetic phases :

At high pH (low proton concentration) the Eadie Hofstee plot is non-linear whereas at low pH-values (high proton concentration) the two phases merge to a single phase (Figure 18 C).

Steady-state kinetics of beef heart oxidase monomers and *P. denitrificans* oxidase monomers are non-linear. They exhibit the same dependence on changes of pH and ionic strength (Figure 18 D).

Detergents affect turnover numbers more than Michaelis constants. The Eadie Hofstee plots were non-linear in all detergents tested.

Positive charges such as H^+ and Na^+ affect the speed of the conformational change of the oxidase in a similar manner. A large amount of such ions speeds up the conformational change, thus increasing the turnover number and increasing the Michaelis constant. The rate of cytochrome c oxidation is limited by the diffusion of cytochrome c in this case. The conformational change of the oxidase becomes slow when only a few positive charges are present, thus introducing the second phase in the steady-state kinetic turnover.

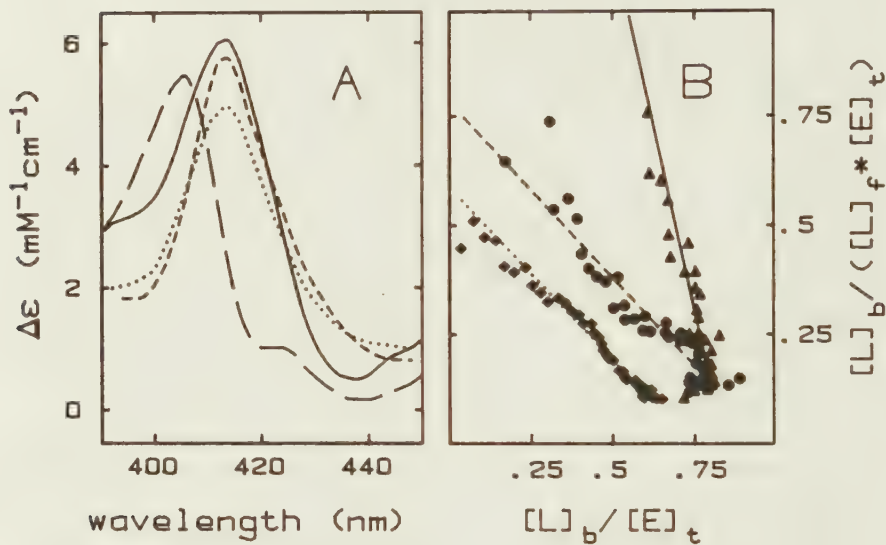


Figure 18: Summary of binding and kinetic experiments

Panel A : Difference spectra for the oxidase ferricytochrome c complexes : (—) in 0.2% Tween-80 instead of laurylmaltoside, (---) dimeric oxidase, (.....) monomeric oxidase, and (- - - -) *P. denitrificans* oxidase. Buffer : 10 mM tris-HCl pH 7.4, 40 mM NaCl, 0.1 % laurylmaltoside, ionic strength 49 mM.

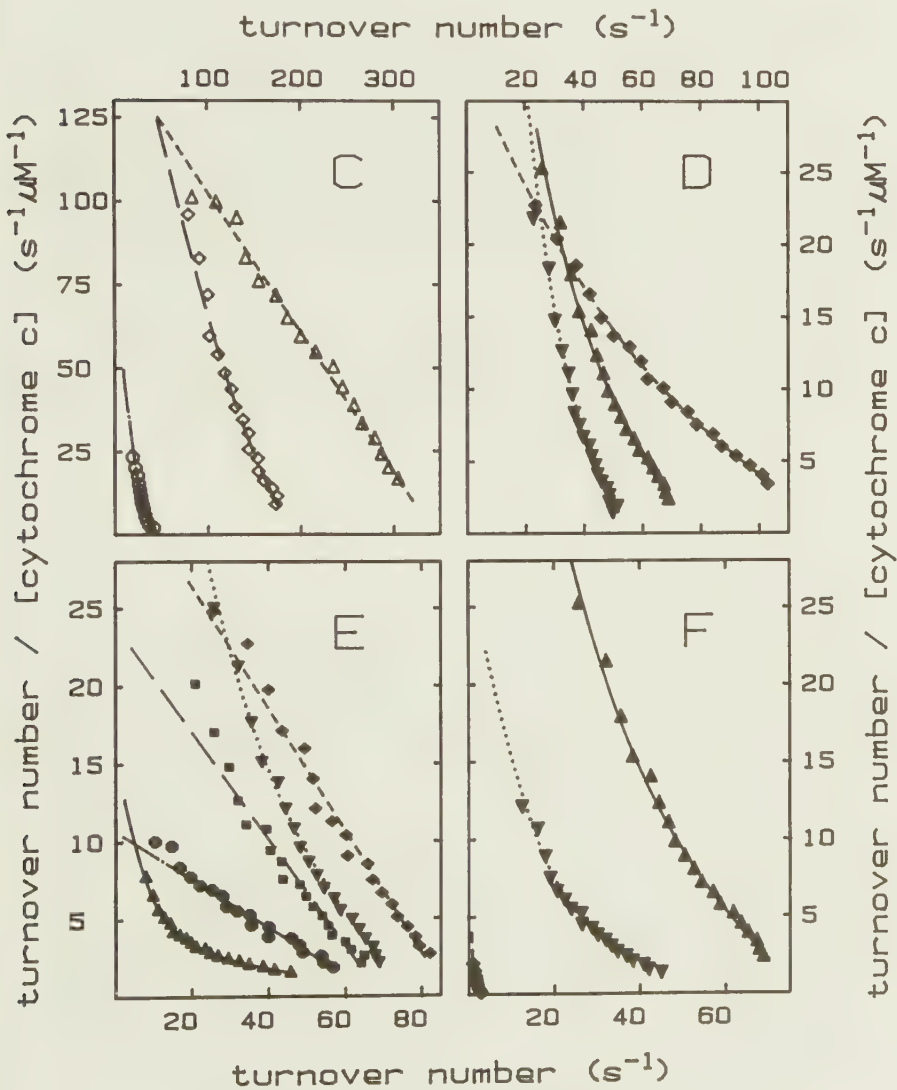
Panel B : Eadie Scatchard transformation of spectroscopic titrations, buffer as in A. Dimeric beef heart oxidase ($\blacktriangle$, —, $K_D=0.3 \mu\text{M}$), monomeric beef heart oxidase ($\bullet$, , $K_D=1.3 \mu\text{M}$), and *P. denitrificans* oxidase ($\blacklozenge$, - - - - - , $K_D=0.97 \mu\text{M}$).

Panel C : Eadie Hofstee transformation of steady-state activity of dimeric beef heart oxidase at different pH-values in tris- and bis-tris-HCl at an ionic strength of 49 mM in 0.1% laurylmaltoside. pH 5.6 (Δ , , $K_{M1}=2.4 \mu\text{M}$), pH 7.4 ($\circ$, — — — , $K_{M1}=0.7 \mu\text{M}$, $K_{M2}=4.4 \mu\text{M}$) pH 8.6 ($\circ$, — — — — , $K_{M1}=0.4 \mu\text{M}$, $K_{M2}=16 \mu\text{M}$).

Panel D : Eadie Hofstee transformation of steady-state activity of different oxidases, buffer as in A. Dimeric oxidase ($\blacktriangle$, —, $K_{M1}=0.54 \mu\text{M}$, $K_{M2}=5.7 \mu\text{M}$), monomeric oxidase (∇ , , $K_{M1}=0.31 \mu\text{M}$, $K_{M2}=5.0 \mu\text{M}$), and *P. denitrificans* oxidase ($\blacklozenge$, - - - - - , $K_{M1}=1.8 \mu\text{M}$, $K_{M2}=11 \mu\text{M}$).

Panel E : Eadie Hofstee transformation of steady-state activity of dimeric beef heart oxidase at varying ionic strengths. ($\blacktriangle$, —) 24 mM, (∇ ,) 49 mM, ($\blacklozenge$, - - - - -) 74 mM, ($\bullet$, — — — —) 99 mM, ($\bullet$, — — — —) 150 mM other conditions as in panel A. The lines drawn through the curves are the simulated curves with the parameters as listed in Table 6.

Panel F : Eadie Hofstee transformation of steady-state activity in different detergents, buffer as in A. ($\blacktriangle$, —, $K_{M1}=0.54 \mu\text{M}$, $K_{M2}=5.7 \mu\text{M}$) 0.1% laurylmaltoside, (∇ , , $K_{M1}=0.71 \mu\text{M}$, $K_{M2}=14 \mu\text{M}$) 0.2% Tween-80, and ($\blacklozenge$, - - - - - , $K_{M1}=0.12 \mu\text{M}$, $K_{M2}=2.7 \mu\text{M}$) 0.2% Triton X-100.



3.4 CD and MCD Difference Spectra of Oxidase with Cytochrome c

3.4.1 CD Difference Spectra : A Probe for Conformational Changes

The CD spectrum of a 1:1 complex of fully oxidized oxidase and ferricytochrome c is significantly different from the sum of the CD spectra of its components (Figure 19 B). The difference CD spectrum has a maximum at 414 nm and a small minimum at 431 nm. The difference signal saturates at a 1:1 ratio of heme c to heme aa₃ (see Table below).

heme c / heme aa ₃	$\Delta[\Theta]$ at 414 nm (mM ⁻¹ cm ⁻¹)	$\Delta(\Delta\epsilon/H)$ at 406 nm (M cm T) ⁻¹
0.38	22'900	1.94
0.80	51'300	4.12
1.13	60'000	5.20
1.49	56'700	4.84
2.20	57'800	5.38

Heme aa₃ was fixed at 4.14 μ M.

Table 9: Spectroscopic titration of oxidase observed by CD and MCD

The difference CD spectrum shown in Figure 19 indicates a large conformational perturbation of ferricytochrome c and a small shift of the heme a CD signal. The minimum of the Soret CD spectrum of free cytochrome c at 417 nm is lost when it binds to the oxidase. This can be observed from the positive difference CD spectrum at this wavelength (Figure 19 B, solid line).

The CD spectrum of a complex of ferrocytochrome c bound to fully reduced oxidase differs significantly from the summed CD spectra of the components (Figure 20 B). The difference spectrum is attributed to ferrocytochrome c alone, because cytochrome c oxidase has no major absorption band below 430 nm (Figure 20 C, dotted line). The maximal ellipticity increase is around 415 nm, as in the oxidized complex. Thus oxidized and reduced oxidase are inducing a similar increase of ellipticity on ferricytochrome c and ferrocytochrome c, respectively. This is an indication of similar conformational changes in both redox states.

3.4.2 MCD Difference Spectra : A Probe for Electronic Changes

CD differences are a probe for conformational rearrangements of the protein matrix. But such changes could as well change the heme electronic structure by affecting iron and porphyrin orbitals. Direct evidence for a change in the heme c electronic structure was

obtained by comparing the MCD spectrum of a 1:1 complex of ferricytochrome c and oxidase with the sum of the spectra of the components (Figure 19 A). A difference MCD spectrum was observed with a maximum at 408 nm and a minimum at 420 nm. The main peak of the MCD spectrum (Figure 19 A, solid line) is caused by a red shift of the heme c derivative shaped MCD signal by 1 nm. The negative band centered around 420 nm is attributed to heme a. The shift of the heme c band is related to a change of electronic structure of heme c. A red shift from 408 to 409 nm can be caused by an aromatic system with an additional conjugated electron. A lesser deformation of the heme plane whereby a better overlap of porphyrin orbitals with the d-orbitals of the iron is achieved could be a further explanation.

In the reduced oxidase - ferrocyanochrome c complex, a red shift of the heme c absorption is observed. Contributions to the difference spectrum below 430 nm by heme a are small since no absorption band of heme a is present in this region (Figure 20 C, dotted line). Both oxidized and reduced CD and MCD difference spectra in the range from 480 to 600 nm were not above noise level.

Figure 19: MCD, CD and absorption spectra and difference spectra of fully oxidized oxidase and ferricytochrome c

Panel A : MCD spectrum of cytochrome c (-----, left ordinate), MCD spectrum of oxidase (....., left ordinate), and MCD difference spectrum (———, right ordinate). Control : difference spectrum with 300 mM NaCl (— · — · —, right ordinate). Buffer : 10 mM tris-HCl pH 7.4, 15 mM NaCl, 0.1% laurylmaltoside.

Panel B : CD spectrum of cytochrome c (-----, left ordinate), CD spectrum of oxidase (....., left ordinate), and CD difference spectrum (———, right ordinate). Control : difference spectrum in the presence of 300 mM NaCl (— · — · —, right ordinate).

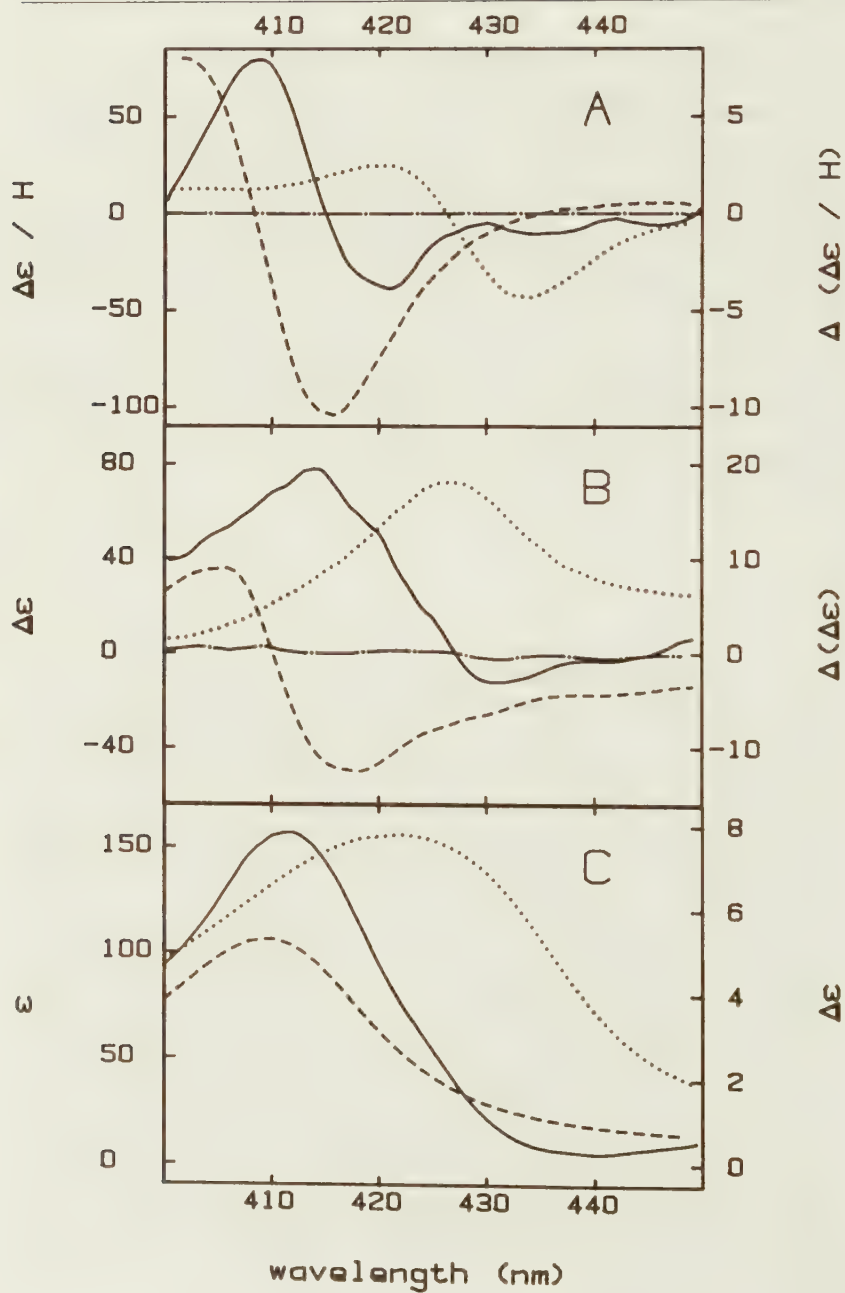
Panel C : Absorption spectrum of cytochrome c (-----, left ordinate), absorption spectrum of oxidase (....., left ordinate), and difference absorption spectrum (———, right ordinate).

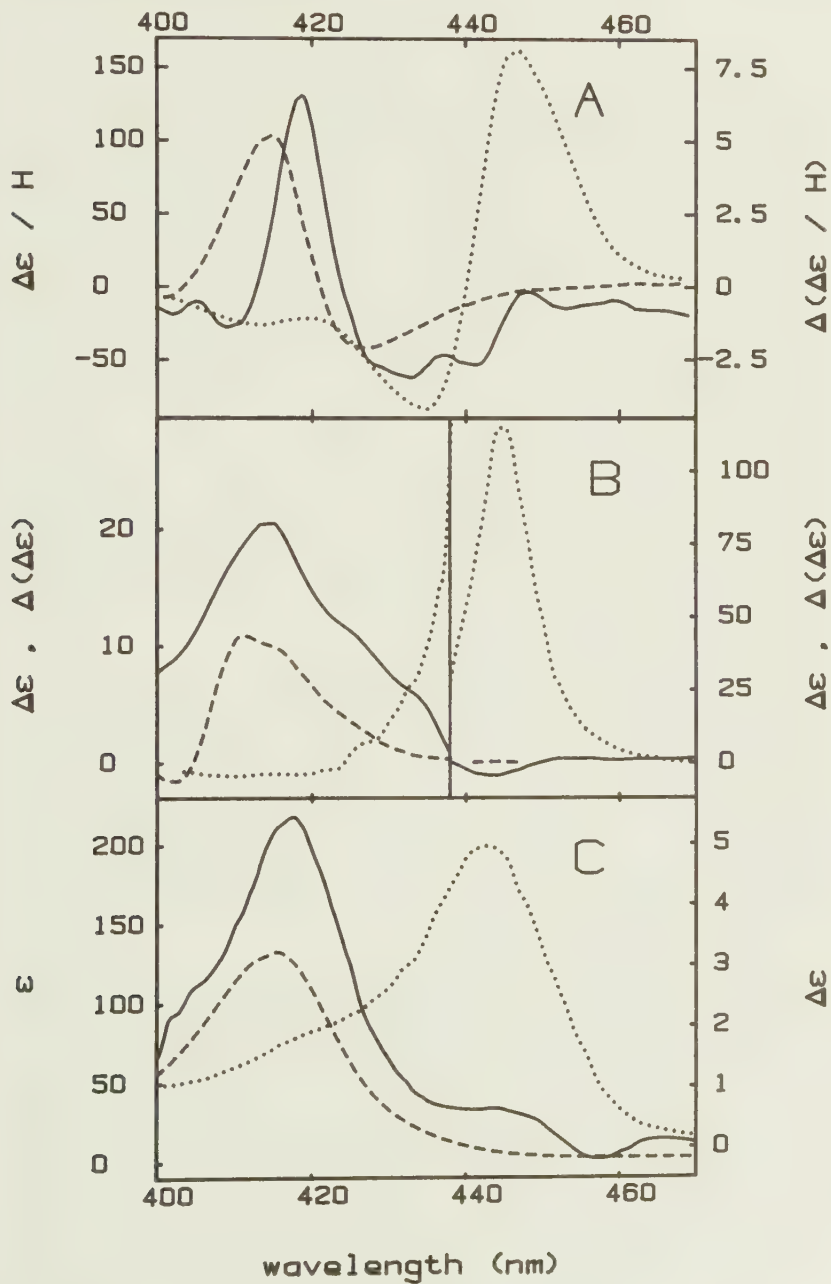
Figure 20: MCD, CD and absorption spectra and difference spectra of fully reduced oxidase and ferrocyanochrome c

Panel A : MCD spectrum of cytochrome c (-----, left ordinate), MCD spectrum of oxidase (....., left ordinate), and MCD difference spectrum (———, right ordinate). Buffer : 10 mM tris-HCl pH 7.4, 15 mM NaCl, 0.1% laurylmaltoside, 5 mM ascorbate, and 60 μ M TMPD.

Panel B : CD spectrum of cytochrome c (-----), CD spectrum of oxidase (.....), and CD difference spectrum (———). The ordinate for the wavelengths below 438 nm is enlarged by a factor of three.

Panel C : Absorption spectrum of cytochrome c (-----, left ordinate), absorption spectrum of oxidase (....., left ordinate), and difference absorption spectrum (———, right ordinate).





3.5 The Nature of the Difference Spectra

As the optical difference spectra caused by binding of cytochrome c to the oxidase are too complex to be fully understood, a few simpler systems have been analysed. It is particularly important to be able to assign the difference signal to either cytochrome c or oxidase. Furthermore, the specificity of the signal had to be tested. In a first step D. Gantner (1986) measured the perturbation of the absorption spectrum of cytochrome c (oxidized and reduced) by solvents and non-heme ligands. A second approach was used by C. Weber (1986) by measuring the perturbation of the absorption and the CD spectrum of oxidase by solvents and non-heme ligands. In a third step the effect of solvents and non-heme ligands on the MCD spectrum of cytochrome c was measured. These measurements included the perturbation of the cytochrome c optical spectra by changes in pH (the alkaline transition, see Introduction).

Use of cytochrome c or oxidase from different species could provide insight into the general features of oxidase - cytochrome c interaction. This approach has been initiated by measuring difference spectra of the beef heart oxidase - chicken cytochrome c, beef heart oxidase - *Candida krusei* cytochrome c and of the *Paracoccus denitrificans* oxidase - horse heart cytochrome c complexes. All perturbations were carried out using the following buffer: 10 mM tris-HCl pH 7.4, 15 mM NaCl, and 0.1% laurylmaltoside.

3.5.1 Optical Spectra of Alkaline Cytochrome c

Optical spectra of horse heart cytochrome c were measured in 10 mM tris-HCl pH 7.4, 15 mM NaCl, and 0.1% laurylmaltoside. The pH was adjusted to 10 with NaOH and another set of optical spectra was recorded. On the basis of CD spectra of oxidized cytochrome c at pH 10, of cytochrome c with extrinsic heme ligands, and of partially denatured cytochrome c, Myer (1968) concluded that the minimum at 417 nm is diagnostic for an intact heme pocket. He suggested that this transition is sensitive to the Met-80 sulfur-iron ligand. A lack of this signal indicates a significant change of the geometry of this bond. In the alkaline form, the heme crevice of cytochrome c is opened, and the 6th coordination site of the heme iron may be occupied by water.

The CD difference spectrum, alkaline minus native, has a similar shape as the CD difference due to the binding of cytochrome c to oxidase. Accordingly, one might assume that oxidase opens the heme crevice of cytochrome c during binding. However, the pronounced similarity between the oxidase bound cytochrome c and the alkaline form of cytochrome c is not supported if the difference absorption and MCD spectra for the cytochrome c - oxidase complex and for alkaline minus native cytochrome c are compared (compare solid line of Figure 19 A with Figure 21 B; solid line of Figure 19 C with Figure 21 F).

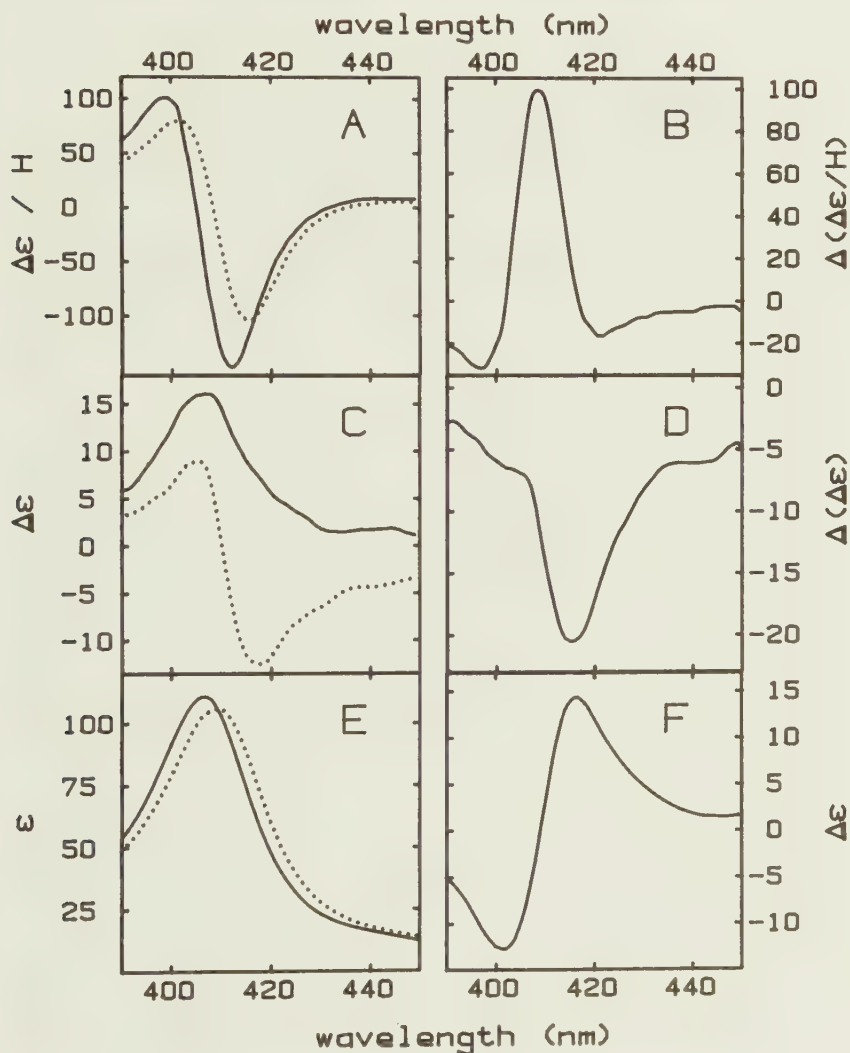


Figure 21: MCD, CD and absorption spectra of alkaline ferricytochrome c

Panel A: MCD spectrum of alkaline cytochrome c at pH 10 (—), MCD spectrum of 'native' cytochrome c at pH 7.4 (·····). **Panel B:** MCD difference spectrum native - alkaline
Panel C: CD spectrum of alkaline cytochrome c at pH 10 (—), CD spectrum of 'native' cytochrome c at pH 7.4 (·····). **Panel D:** CD difference spectrum native - alkaline
Panel E: Absorption spectrum of alkaline cytochrome c at pH 10 (—), absorption spectrum of 'native' cytochrome c at pH 7.4 (·····). **Panel F:** difference spectrum native - alkaline

3.5.2 Perturbation of Heme c Optical Spectra by Solvents

Primary alcohols perturb the optical spectrum of ferricytochrome c. The difference spectrum is maximal at 402 nm and minimal at 416 nm (Figure 22 F, solid line). The shape of the spectrum in the Soret region is similar to the ferricytochrome c - ferrocytochrome c difference spectrum. But in the α -band the effect of these alcohols is opposite (Gantner, 1984). Polyalcohols perturb the absorption spectrum in a different manner : The difference spectrum is maximal at 410 to 412 nm without a minimum (Figure 22 F, dashed line). The spectrum is thus similar to that for the binding of ferricytochrome c to phosvitin or to oxidase.

As absorption spectra do not give information about protein conformation and electronic structure, the perturbation of MCD and CD of cytochrome c by a primary and a secondary alcohol, by phosvitin, glycine, and NaCl was measured.

The MCD results confirm the observations made in the absorption spectra. Primary alcohols are able to enter into the heme pocket and affect the heme iron electronic structure. The lack of a CD difference spectrum with ethanol proves that slight changes of solvent polarity have no profound effects on the three dimensional structure of cytochrome c.

The MCD perturbation spectrum of ferricytochrome c with ethyleneglycol was maximal at around 410 nm, (Figure 22 B, dotted line). Perturbation with 10% ethanol resulted in a shift of the derivative shaped heme c MCD signal to the blue. This corresponds to cytochrome c with a proportion of 10-20% in the alkaline form (Figure 22 B, solid line). The CD spectrum remained unchanged during the perturbation with ethanol. Raising the ionic strength with sodium chloride caused a negative band at 412 nm. Otherwise NaCl did not change the MCD spectrum. The CD difference spectrum was only partially above noise level at 412 nm (Figure 22 C, solid line).

Mixing ferricytochrome c with 50 mM glycine, an amino acid with a very strong dipole moment, caused an MCD difference spectrum with a large negative band at 408 and a positive band around 416 nm (Figure 22 A, dotted line). The CD difference is less pronounced with a maximum at 416 nm (Figure 22 C, dotted line).

Binding of phosvitin to ferricytochrome c resulted in a difference MCD spectrum that was maximal at 410 nm and negative below 400 nm. The difference spectra obtained with phosvitin are dependent on the ratio of cytochrome c to phosvitin, because one phosvitin molecule is able to bind 10 cytochrome c molecules. The spectroscopic behaviour of these binding sites is inhomogeneous. The CD difference spectrum is maximal at 407 nm.

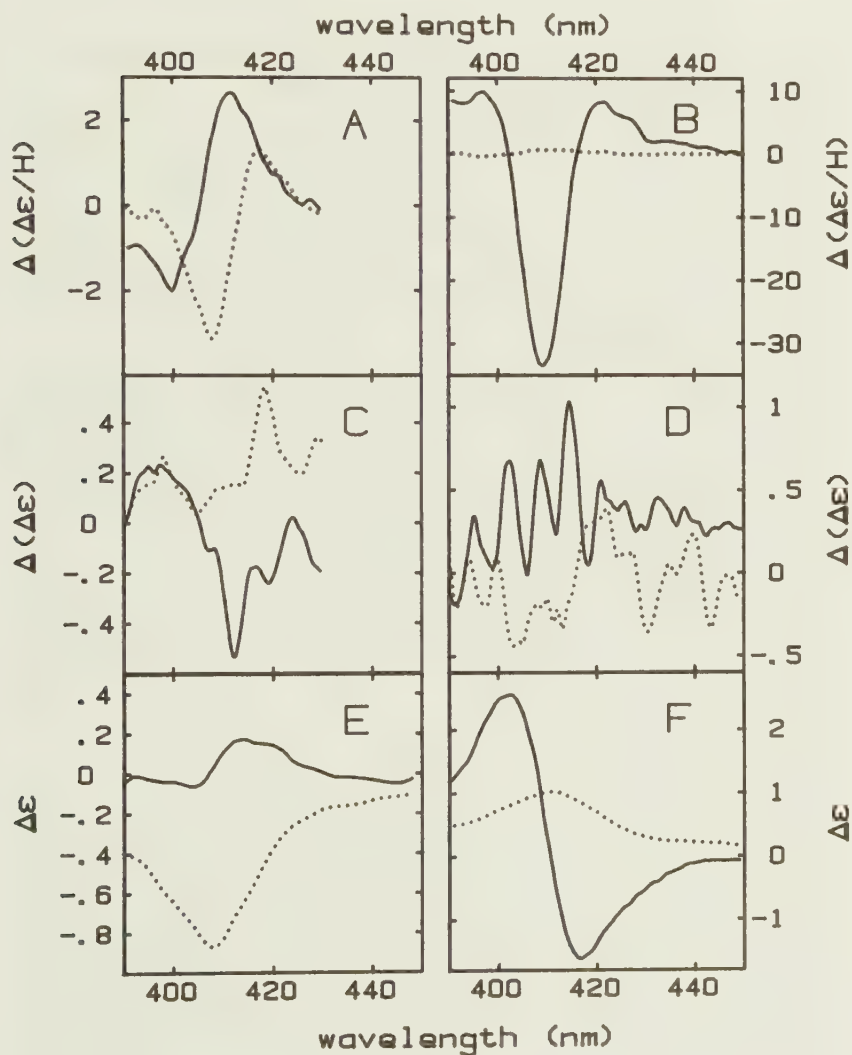


Figure 22: Perturbation of ferricytochrome c

Panel A, C, and E : MCD, CD, and Absorption perturbation spectra of ferricytochrome c with (—) 100 mM NaCl, (·····) 50 mM Glycine.

Panel B, D, and F : MCD, CD, and Absorption perturbation spectra of ferricytochrome c with (—) 10% (v/v) ethanol, (·····) 5% (v/v) ethyleneglycol.

3.5.3 Binding and Kinetics of Cytochromes c from Other Species

a) Chicken cytochrome c

Binding of chicken flight muscle cytochrome c to oxidase induces a similar difference spectrum as horse heart cytochrome c. The difference spectrum is maximal at 410 nm with an extinction coefficient of $7 \text{ mM}^{-1} \text{ cm}^{-1}$. Binding can be titrated yielding a ratio of 1 heme c bound per heme a₃. The affinity of oxidase for chicken cytochrome c is $0.09 \mu\text{M}$ at 24 mM ionic strength. Steady-state turnover at 24 mM was biphasic ($K_{m1}=0.05 \mu\text{M}$, $\text{TN}_{\text{max}1}=10.6 \text{ s}^{-1}$, $K_{m2}=11.9 \mu\text{M}$, $\text{TN}_{\text{max}2}=73 \text{ s}^{-1}$). As with horse heart cytochrome c it became monophasic at 150 mM ionic strength ($K_m=5.9 \mu\text{M}$, $\text{TN}_{\text{max}}=81.6 \text{ s}^{-1}$).

b) *Candida krusei* cytochrome c

Binding of *C. krusei* cytochrome c induces a difference spectrum with a maximum at 404 nm ($\Delta\epsilon=6 \text{ mM}^{-1} \text{ cm}^{-1}$). Binding was titrated yielding a ratio of 1 heme c bound per heme a₃. The affinity of beef heart oxidase for *C. krusei* cytochrome c was $0.07 \mu\text{M}$ at 24 mM ionic strength. Steady-state turnover at 24 mM was biphasic ($K_{m1}=1.2 \mu\text{M}$, $V_{\text{max}1}=22 \text{ s}^{-1}$, $K_{m2}=38 \mu\text{M}$, $V_{\text{max}2}=78 \text{ s}^{-1}$). As with horse heart cytochrome c it became monophasic at 150 mM ionic strength ($K_m=5.3 \mu\text{M}$, $V_{\text{max}}=79 \text{ s}^{-1}$).

These results show that conservative mutations do not change the affinity of the cytochrome c for beef heart oxidase. The difference spectra, however, were quite different in magnitude and peak position for the *C. krusei* cytochrome c. This indicates that the heme is a very sensitive marker for the binding and unlocking mechanism. The kinetic parameters are more affected, particularly the high affinity K_m becomes weaker in the case of *C. krusei* cytochrome c.

3.6 Modified Horse Heart Cytochromes c

The method of choice for the localization of the structures responsible for binding of cytochrome c or for electron transfer is site directed mutagenesis. With this procedure particular residues that are expected to play an important role in electron transfer are changed on the DNA-level. The gene is incorporated in a plasmid, cloned, expressed and the properties of the modified cytochrome c are measured. Such studies, however, are very time consuming. For that reason, the chemical modification of horse heart cytochrome c is apt to provide a few insights, at a shorter term. Most chemical modifications, however, are less specific, usually only an entire group of residues is modified as a whole. Another type of modification consists in the introduction of chain breaks with proteases. These act quite specifically on certain bonds. By chain breaks the molecule is made more flexible. It is thus possible to test structural requirements of electron transfer by studying such 'floppy' derivatives.

3.6.1 Surface Modified Cytochromes c

An important feature of the electron transfer complex is the proper orientation of groups. This orientations can be obstructed by fixing bulky groups to the binding site of cytochrome c. The most suitable groups for this purpose are the 19 lysine residues of cytochrome c, 6 - 8 of which are reported to be involved in the binding site. Analysis of the kinetic and binding parameters of a set of differently lysine modified cytochromes c has been performed in a cooperation with C.J.M. Wallace and A. Proodfoot (University of Geneva).

Binding of these cytochromes was titrated spectrophotometrically in 10 mM tris-HCl pH 7.4, 15 mM NaCl, and 0.2% Tween-80. Steady-state activity was measured at low ionic strength ($\mu = 25$ mM) and at high ionic strength ($\mu = 150$ mM). The low ionic strength buffer was identical with the binding buffer. The high ionic strength buffer contained additional 140 mM NaCl.

a) 19-N- ϵ acetimidyl cytochrome c

Acetimidylation of all lysines neither changes the charge nor introduces a bulky group. The difference spectrum due to binding to oxidase is native-like in shape with a 10-15% smaller magnitude (Figure 23 A, solid line). The dissociation constant is 0.21 μ M compared to 0.15 μ M for native horse heart cytochrome c. The high affinity Michaelis constant and maximal turnover number velocity are unchanged, the low affinity K_m however is three times larger than with native cytochrome c (Table 10).

b) 19-N- ϵ -isopropyl cytochrome c

The bulky, hydrophobic isopropyl residues reduce the affinity to oxidase by a factor of four (Table 10). The difference spectrum due to binding to oxidase is smaller, indicating a less efficient overlap of the oxidase orbitals with the orbitals of heme c (Figure 23 A, dotted line).

c) 19-N- ϵ -dimethyl cytochrome c

The affinity of the dimethyl derivative is reduced by a factor of six (Table 10). The two methyl groups close to the nitrogen obstruct ionic interactions more than a single isopropyl group because the latter can be bent away more easily. Therefore, the difference spectrum for the dimethyl derivative is smaller than for the isopropyl derivative (Figure 23 A, dashed line). Steady-state turnover at high ionic strength is strongly affected: The TN_{max} is three times lower, the K_m is more than 10 times weaker. At low ionic strength, however, the high affinity turnover is increased.

d) 1-O-acetyl cytochrome c / 2-O-acetyl cytochrome c

Modification of tyrosine 67 (1-O-acetyl Figure 23 B, solid line) or modification of tyrosine 67 plus tyrosine 85 (2-O-acetyl, Figure 23 B, dotted line) makes the derivatives auto-oxidable. The difference spectra due to the binding to fully oxidized oxidase are not changed in these derivatives, but they have a slightly better affinity for oxidase (Table 10).

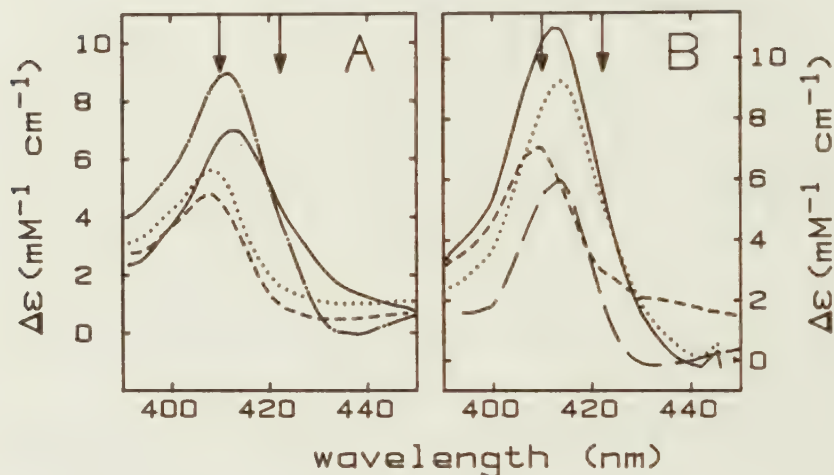


Figure 23: Difference spectra of surface modified cytochromes c

Panel A : (————) 19-N- ϵ -acetimidyl cytochrome c, (·····) 19-N- ϵ -isopropyl cytochrome c, (-----) 19-N- ϵ -dimethyl cytochrome c, (— · — · —) native cytochrome c.
Panel B : (————) 1-O-acetyl cytochrome c (tyrosine 65 modified), (·····) 2-O-acetyl cytochrome c (tyrosine 65 and 87 modified), (-----) 19-N- ϵ -guanido cytochrome c, (— · — · —) 19-N- ϵ -citraconyl cytochrome c.

e) 19-N- ϵ -guanido cytochrome c

The guanido derivative has an affinity and a difference spectrum comparable to that of the isopropyl derivative (Figure 23 B, dashed line). The most striking difference is a three fold lower Michaelis constant and an increased TN_{max} in the steady-state assay at high ionic strength (Table 10).

f) 19-N- ϵ -Citraconyl cytochrome c

No steady-state assays could be performed with this derivative as it was not soluble at a sufficient concentration. The difference spectrum due to the binding to oxidase was slightly smaller and shifted 2 nm to the red when compared with native horse heart cytochrome c. The affinity of this derivative is roughly identical with that of native cytochrome c.

3.6.2 Non-Covalent Cytochrome c Complexes

The cleavage of acetimidylated cytochrome c with trypsin occurs predominantly at a single peptide bond forming two fragments, fragment (1-38) and fragment (39-104). They can be combined to a stable complex that exhibits physical properties similar to the intact protein. The 19-N- ϵ -acetimidyl 1-37:38-104 non-covalent complex was prepared by carboxypeptidase B digestion of the N- ϵ -acim fragment 1-38, and semisynthetic elongation of the N- ϵ -acim 39-104 fragment (Proudfoot et al., 1986). Trypsin has been used under different sets of conditions to prepare the 1-55:56-104 two-fragment complex. The 1-50:51-104 non-covalent complex was prepared by limited acid hydrolysis optimizing the yield of fragments 1-50 and 51-104 (Wallace and Proudfoot, 1987).

The non-covalent complexes 1-50:51-104, 1-55:56-104, and 1-38:39-104 exhibit blue shifted absorption maxima at pH 7.4. They are known to have lower pK values for the alkaline transition (Wallace and Proudfoot, 1987). Binding to oxidase in 10 mM tris-HCl pH 7.4, 15 mM NaCl, and 0.2% Tween-80 results in a larger difference spectrum than with native cytochrome c. The difference spectra of these complexes are similar to the native minus alkaline difference spectrum of horse heart cytochrome c. The magnitude of the difference at 417 nm is proportional to the part of the complex that is already in the alkaline form at pH 7.4. The pK of the alkaline transition is higher for the acetimidylated derivatives. Only 15% of the acim 1-38:39-104, and 17% of the acim 1-37:38-104 cytochrome c is in the alkaline form at pH 7.4. Panel A and Panel B of Figure 24 show the difference spectra that have been measured during the spectroscopic titration at pH 7.4. The difference signal of the 1-50:51-104 non-covalent complex extrapolates to $16.7 \text{ mM}^{-1}\text{cm}^{-1}$. The pK of the alkaline transition of this derivative is 7.35. This means that at pH 7.4 about 53% of the molecules are in the alkaline form. The maximal difference for native minus alkaline horse heart cytochrome c is minus $20 \text{ mM}^{-1}\text{cm}^{-1}$ (Figure 21 F). The correction at 416 nm for 53% is now minus $10.6 \text{ mM}^{-1}\text{cm}^{-1}$. The corrected spectrum is plotted in Figure 24 (Panel C, solid line). This procedure can be applied to all non-covalent derivatives (Figure 24, panel C and D).

The absolute spectra of the non-covalent complexes are blue shifted and are similar to the spectrum of alkaline horse heart cytochrome c. An attempt to correct the spectra of the non-covalent derivatives for their partial content of alkaline forms was undertaken. Subtraction of the alkaline minus native spectrum of horse heart cytochrome c did not yield a native spectrum. The resulting difference shows the amount of additional disruption of the heme configuration that has been introduced by the chain break.

The dissociation constant of the 1-38:39-104 non-covalent complex with oxidase is the same as with the unmodified enzyme. The other non-covalent derivatives have dissociation constants not far different from native cytochrome c.

Cytochrome c oxidase imposes a conformation onto the non-covalent derivatives with a 'near native' electronic structure of the heme c. This is an unexpected result as the dis-

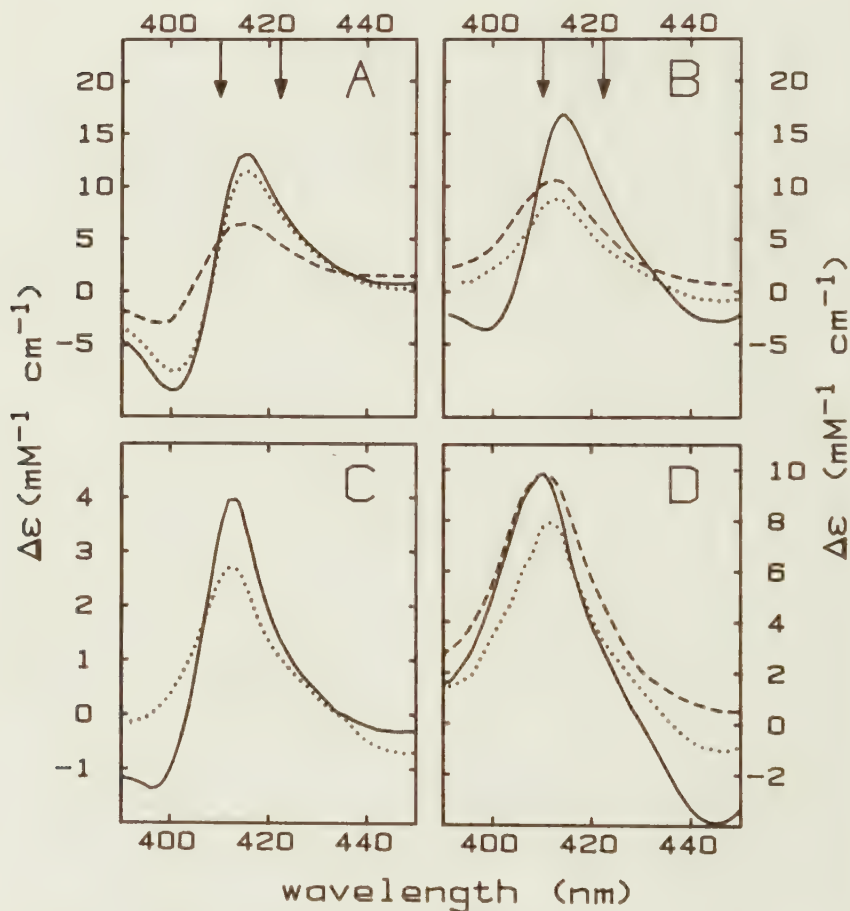


Figure 24: Difference spectra of cytochrome c with a cleaved polypeptide chain

Panel A : (—) 1-50:51-104 non-covalent cytochrome c complex, (·····) 1-55:56-104 non-covalent cytochrome c complex, (-----) 3-104 cytochrome c.

Panel B : (—) 1-38:39-104 non-covalent cytochrome c complex, (·····) 19-N- ϵ -acetimidyl 1-38:39-104 non-covalent cytochrome c, (-----) 19-N- ϵ -acetimidyl 1-37:38-104 non-covalent cytochrome c.

Panel C : (—) 1-50:51-104 corrected for 53% alkaline form, (·····) 1-55:56-104 corrected for 59% alkaline form.

Panel D : (—) 1-38:39-104 corrected for 67% alkaline form, (·····) 19-N- ϵ -acetimidyl 1-38:39-104 corrected for 15% alkaline form, (-----) 19-N- ϵ -acetimidyl 1-37:38-104 corrected for 17% alkaline form.

sociation constants of the oxidase with these derivatives are not affected. This means the energy necessary to pull the derivatives into a native-like conformation is small compared to the energy necessary for the conformational change that oxidase imposes on cytochrome c in order to prepare it for electron transfer.

Steady-state activity of oxidase with these non-covalent derivatives was measured at low ionic strength 10 mM tris-HCl pH 7.4, 15 mM NaCl, and 0.2% Tween-80 (ionic strength = 24 mM) and at high ionic strength (same buffer but 140 mM NaCl, ionic strength = 149 mM).

A major problem preventing the accurate determination of kinetic constants at pH 7.4 was the partial alkaline transition and the fast autooxidizability of these derivatives. For the non-acetimidylated derivatives 1 - 38 : 39 - 104, 1 - 50 : 51 - 104, and 1 - 55 : 56 - 104 the measurements should be repeated at a lower pH (5.5 to 6.5). As with native cytochrome c the experimental data could be fitted best to equation 2. The results of these fits are compiled in Table 10.

Steady-state activity at low ionic strength was biphasic for all these derivatives except for the 3 - 104 cytochrome c. The high affinity Michaelis constant was found to be similar for all of the derivatives (approximately 0.2 μ M - 0.5 μ M). This is the same range as the dissociation constant that has been determined by the spectroscopic titration. The maximal velocity for the high affinity phase is similar for these derivatives. Generally it seems that the high affinity site is not affected by cleavage of the polypeptide chain at position 37, 38, 50, or 51.

The effect on the low affinity phase is neither very pronounced. The very low velocity of the acim 1 - 37 : 38 - 104 derivative could have been caused by the large content of oxidized cytochrome c (due to the alkaline transition) in the stock solution. It seems advisable therefore, to repeat this determination at a lower pH.

Steady-state activity at high ionic strength was monophasic. All modifications led to an increase of the Michaelis constant and to a decrease of maximal turnover. This fact can be explained by the increased energy of activation that is used to pull these derivatives into the proper configuration for electron transfer. Due to problems caused by autooxidation and alkaline transition, it was not possible to assign a 'degree of floppyness' to the different derivatives. The most pronounced difference from unmodified cytochrome c is observed for the 3 - 104 cytochrome c. This derivative, lacking the first two amino acids (a Gly and a conservative Asp) shows monophasic turnover at low ionic strength ! The Michaelis constant is two times larger than the low affinity Michaelis constant of native cytochrome c. The maximal turnover number is approximately three times lower. This large difference cannot be explained by the slightly lower affinity of this derivative for the oxidase. However, contamination by denatured or partially denatured cytochrome c cannot be ruled out.

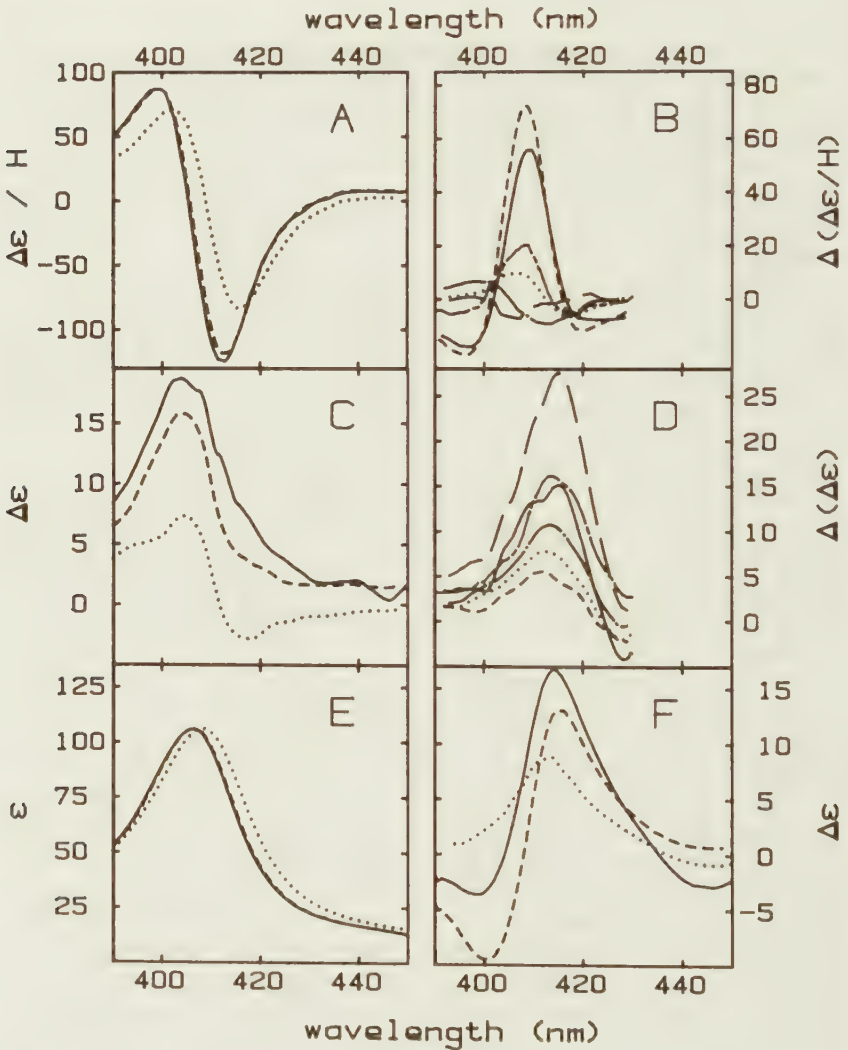


Figure 25: MCD, CD and absorption spectra of cytochrome c with a cleaved polypeptide chain

(—) 1-38:39-104 cytochrome c, (·····) acim 1-38:39-104 cytochrome c
 (-----) 1-50:51-104 cytochrome c.

Panel A, C, and E: Absolute spectra.

Panel B, D, and F: Difference spectra due to binding to oxidase; difference spectra corrected for the partial alkaline denaturation: (— — —) 1-38:39-104 cytochrome c, (— · — · —) acim 1-38:39-104 cytochrome c (— — —) 1-50:51-104 cytochrome c.

3.6.3 MCD and CD Difference Spectra of Modified Cytochromes c

The MCD difference signal for the binding of oxidase to the 1-38:39-104 non-covalent cytochrome c has a maximum at 408 nm. The magnitude of this signal is 10 times larger than for the native cytochrome c (Figure 25 B, solid line). Like in the absorption spectrum the difference can be corrected for partial alkaline denaturation (Figure 25 B, long dashes).

The CD difference signal of this derivative is slightly smaller than for the native cytochrome c (Figure 25 D, solid line). If the same alkaline-native renaturation spectrum is subtracted from this difference spectrum, the corrected spectrum is roughly similar to the CD difference spectrum that is observed for native cytochrome c.

The MCD difference spectrum for the binding of oxidase to the 1 - 50 : 51 - 104 non-covalent cytochrome c can be corrected in the same way (Figure 25 B, dashed and dash-dotted line). After a correction, the CD spectrum of this derivative is also much closer to the native difference spectrum (Figure 25 D, dashed and dash-dotted line).

The MCD difference spectrum of the acim 1 - 38 : 39 - 104 is much smaller than that of the non-acim derivative. It is even smaller than that of native cytochrome c. This observation can be explained by the inability of the oxidase to pull cytochrome c to the native conformation when a direct access to the surface lysines is not possible. This observation is supported by the much smaller CD difference spectrum of this derivative.

Both, CD and MCD difference spectra of the permethyl derivative are much smaller than the native difference spectrum. Here, the binding and the proper orientation of groups important for electron transfer is obstructed by the two bulky methyl residues. This is in accordance with the lowered affinity of this derivative for oxidase (Figure 26 B and D, dotted line).

The MCD difference spectrum that is observed for the 3 - 104 derivative is roughly four times larger than for native cytochrome c (Figure 26 B, solid line). In contrast, the CD difference is only half as large (Figure 26 D, dotted line). The most prominent difference is observed in the absorption spectrum, (Figure 26 E, solid line). Here the difference spectrum has a typical derivative shape. This means that the absorption band is shifted to the red by 1 to 2 nm. This is surprising as this shift is smaller in the MCD spectrum (Figure 26 B, solid line).

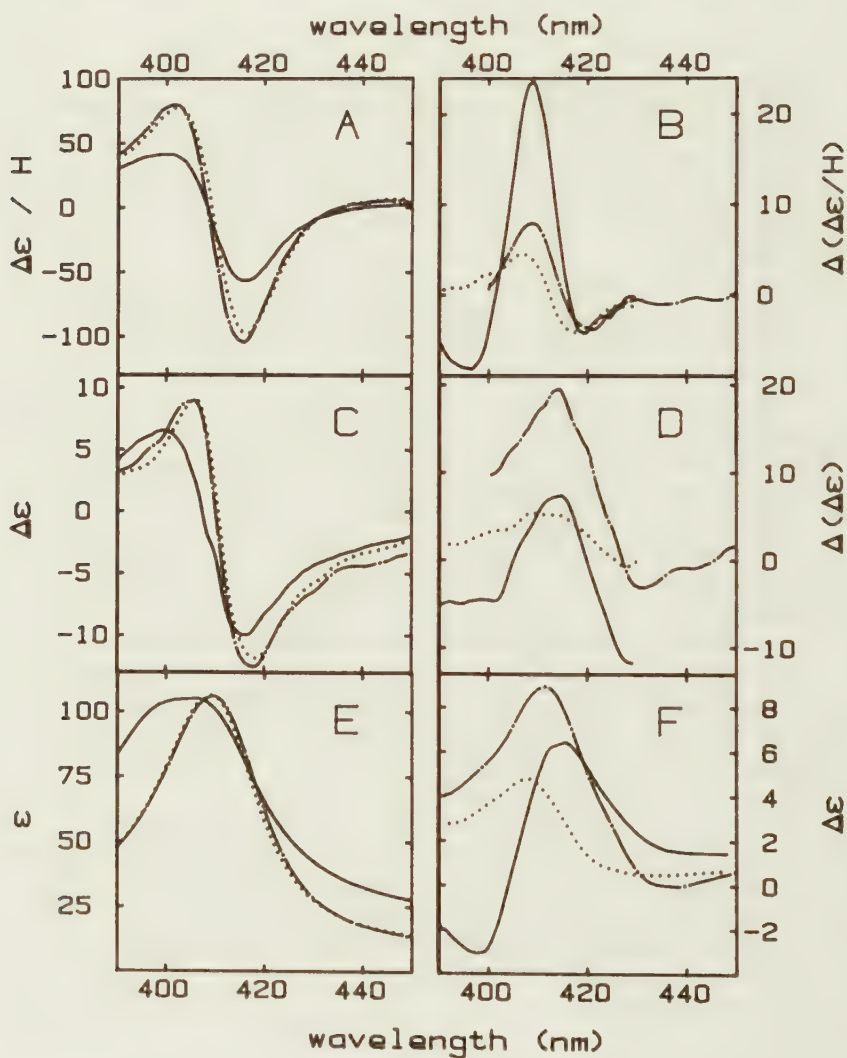


Figure 26: MCD, CD and absorption spectra of surface modified cytochromes c
 (.....) 19-Nεdimethyl cytochrome c, (—) 3-104 cytochrome c, (— · — · —) native cytochrome c

Panel A, C, and E: Absolute spectra.

Panel B, D, and F: Difference spectra due to binding to oxidase.

3.6.4 Chloramine-T Modified Horse Heart Cytochrome c

The two products from the reaction of horse heart ferricytochrome c with chloramine-T, the F-II and F-III CT-cytochromes, contain modifications of the methionines to methionine sulfoxides, but they are distinct in their physiological functions. The ferric and ferrous CT-cytochromes above pH 7.5 have similar spectral properties typical of low-spin hexacoordinate hemes, but distinct from those of the unmodified protein. The ferric spectrum lacks the 695 nm band, the marker of an intact iron-sulfur coordination. Both CT-ferric cytochromes are less stable in the acid region and convert to high-spin forms with a distinct set of pK_a 's. The overall conformation is nearly identical to that of the native protein, but is less stable to thermal unfolding. The two functionally distinct CT-cytochromes are two conformational isomers. The sulfur of methionine-80 is replaced by the oxygen of Met 80 sulfoxide in alternate R or S configuration (Pande et al., 1987).

The CD spectrum of the CT-cytochromes lacks a negative ellipticity at 417 nm with a concurrent increase of ellipticity of the Soret Cotton effect at 406 nm. Such a change in the Soret CD spectrum has been interpreted to represent disruption of heme - polypeptide interactions, the loosening of the heme crevice (Myer and Pande, 1978; Myer, 1985).

Methionine 65 and methionine 80 were oxidized to their sulfoxides as described in Methods. The MCD, the CD and the absorption spectra of a 1:1 complex of oxidase with the F-II, F-III and F-IV derivatives were again significantly different from the sum of the spectra of the components (Figure 27). The F-III- derivative gave rise to a large absorption difference spectrum with a minimum at 396 nm and a maximum at 416 nm (Figure 27 F, dotted line). The part around 410 nm is similar to the difference spectrum of native beef heart cytochrome c. Below 400 nm there is a trough that is not seen in the native case. The CD difference spectrum is smaller at 414 nm and more negative at 406 nm, but the shape is similar to native cytochrome c (Figure 27 D, dotted line).

The largest difference is observed in the MCD difference spectrum. The maximum is shifted to 414 nm with a magnitude 3 times larger than for native cytochrome c. This means that the derivative shaped MCD absorption is shifted approximately 4 nm to the red (Figure 27 A,B, dotted line). The negative part around 420 nm that is observed in the native difference spectrum can not be observed with the F-II, F-III and F-IV derivatives.

The absorption difference spectrum of the F-II derivative is almost zero above 410 nm. The negative peak at 396 nm is comparable to the peak observed for the F-III derivative (Figure 27 F, dashed line). The CD difference spectrum is only half as large at 416 nm as native cytochrome c. The other features resemble the F-III CD difference spectrum (Figure 27 D, dashed line).

The MCD of all derivatives are almost identical but the difference is three to four times larger than for native cytochrome c. The absolute MCD spectrum is shifted by 4 nm to the blue compared to native cytochrome c. This difference is diminished to about 1 to 2 nm in the bound methionine modified cytochromes compared to bound native cytochrome c. This is the result of a large change in electronic structure around the heme iron reverting

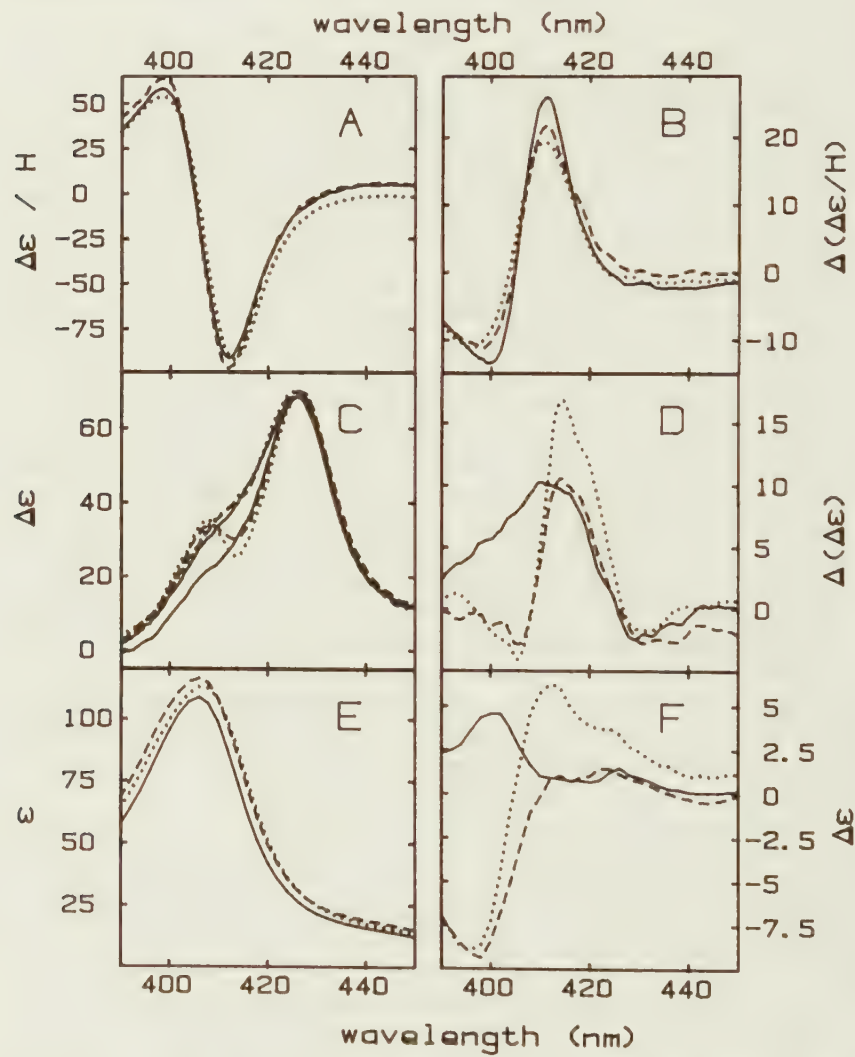


Figure 27: MCD, CD and absorption spectra of chloramine-T modified cytochromes c

(——) F-IV, (·····) F-III, and (-----) F-II.

Panel A : MCD absolute spectra

Panel B : MCD difference spectra of a 1:1 complex of beef heart oxidase with F-II,F-III, and F-IV CT-modified cytochromes c.

Panel C : CD absolute spectra of complex and sum of spectra Panel D : CD difference spectra

Panel E : Absorption spectra Panel F : Absorption difference spectra

the disruption of the native structure caused by the sulfoxide. The F-IV derivative has a absorption difference spectrum with a maximum at 404 nm with an extinction coefficient of $4 \text{ mM}^{-1}\text{cm}^{-1}$. As for the other derivatives, the absorption maximum is centered around 406 nm (Figure 27 E, solid line). The change in conformation is closely similar to native cytochrome c. F-IV is certainly different from native cytochrome c.

3.6.5 Summary of Results from Modified Cytochromes c

The binding of cytochrome c to oxidase is very robust. Cytochromes from a wide range of species bind to beef heart oxidase with only minor changes in K_d . The difference spectrum does not change very much, it is always positive with a maximum at 410 to 414 nm. A larger change is observed when *P. denitrificans* oxidase binds to horse heart cytochrome c. In this case, the maximum difference signal is 406 nm. It is unlikely that the difference spectrum is due to the oxidase since the absorption of *P. denitrificans* is low around 406 nm. Binding of *C. krusei* cytochrome c to oxidase caused a difference spectrum with a maximum at 406 nm. Dimethyl- and isopropyl - modified cytochromes had maxima at 407 and 405 nm. These cytochromes bind with a lower affinity than native horse heart cytochrome c.

Common to all these pairs with a blue shifted smaller difference spectrum is their weaker affinity. It seems possible, therefore, that the binding rearrangements are arrested at an earlier stage, where the heme vicinity is still more polar than in the case of the beef heart oxidase horse heart cytochrome c pair.

Binding of non-covalent cytochrome c to oxidase results in significantly altered difference spectra compared to native cytochrome c. This is explained by a partial alkaline transition of these samples. Based on the pK value for the alkaline transition of these derivatives, the difference spectra can be corrected, resulting in spectra closely similar to that of native cytochrome c bound to oxidase. This can be interpreted to indicate that oxidase imposes a 'near native' conformation and electronic structure on the 'floppy' non-covalent derivatives.

No satisfactory correction is possible for acetimidylated non-covalent derivatives. Presumably, acetimidyl groups around the heme edge prevent the oxidase from inducing a 'near native' conformation on the 'floppy' non-covalent derivatives. It should be noted, however, that acetimidylation alone does not significantly change the difference absorption spectrum for binding to oxidase (Figure 23 A, solid line).

	auto-oxid.	absolute spectrum oxidized and reduced			binding of Cyt c	
cytochrome c derivative	%	oxid. λ (nm)	red. λ (nm)	red./ox. ratio	oxid. λ (nm)	$\Delta\epsilon$ mM ⁻¹ cm ⁻¹
native (horse heart)	<0.001	409.5	415.3	1.250	414	8.6
3-104	0.05	405.3	415.7	1.105	415	5.7
1-38, 39-104	0.86	408.0	415.7	1.216	415	19
1-38, 39-104 paracetimidyl	0.495	406.5	416.0	1.136	414	9.8
1-37, 38-104 paracetimidyl	0.46	408.8	416.0	1.205	414	11.2
1-50, 51-104	small	406.8	415.9	1.162	414	16.7
1-55, 56-104	nd	406.8	416.5	1.206	415	14.7
paracetimidyl	0.033	409.0	415.3	1.106	414	7.7
perisopropylamino	0.012	409.0	415.3	1.215	414	6.25
perdimethylamino	0.014	409.3	nd	nd	414	5.5
parguanido	0.014	409.3	415.0	1.193	409	9.3
percitraconyl	nd	407.0	416.5	1.010	416	6.8
1-0-acetyl	0.24	409.0	415.8	1.213	416	10.3
2-0-acetyl	0.35	408.0	416.0	1.265	416	8.9
Candida krusei	<0.001	408.8	415.8	1.150	406	4.8

	steady-state kinetics of cytochrome c oxidation						binding of Cyt c	
cytochrome c derivative	ionic strength 25 mM						25 mM	
	K_M (μ M)	V_{max} s ⁻¹	K_M (μ M)	V_{max} s ⁻¹	K_M (μ M)	V_{max} s ⁻¹	N_{obs}	K_d (μ M)
native (horse heart)	0.43	7.2	27.0	54.0	6.7	73.0	0.95	0.15
3-104	-----	-----	18.1	26.9	nd	nd	0.94	0.35
1-38, 39-104	-- *	-- *	-- *	-- *	-- *	-- *	1.04	0.21
1-38, 39-104 paracetimidyl	0.24	10.9	10.1	68.7	-- *	-- *	0.92	0.16
1-37, 38-104 paracetimidyl	0.5	10.3	7.4	14.4	26.8	36.5	0.98	0.18
1-50, 51-104	0.19	15.8	6.7	38.9	13.0	56.4	0.82	0.53
1-55, 56-104	0.31	13.4	6.5	56	8.0	47	1.5	0.45
paracetimidyl	0.26	10.6	31.0	35.0	18.3	61.4	0.94	0.21
perisopropylamino	0.53	14.8	13.0	39.8	32.6	89.7	0.90	0.60
perdimethylamino	0.21	47.8	4.3	43.1	71.6	26.9	0.80	0.93
parguanido	0.23	5.3	19.4	29.4	2.3	101	1.17	0.56
percitraconyl	-- +	-- +	-- +	-- +	-- +	-- +	0.92	0.06
1-0-acetyl	0.17	3.7	8.7	29.9	-- *	-- *	0.87	0.08
2-0-acetyl	.004	0.7	22.0	9.3	-- *	-- *	0.96	0.10
Candida krusei	1.24	21.7	38.8	78.6	5.1	91.5	0.82	0.07

Table 10: Summary of binding and kinetic properties of modified cytochromes c

Upper Table : Spectroscopic properties.

Lower Table : Kinetic properties.

+ not soluble, * autooxidation too fast, nd not determined.

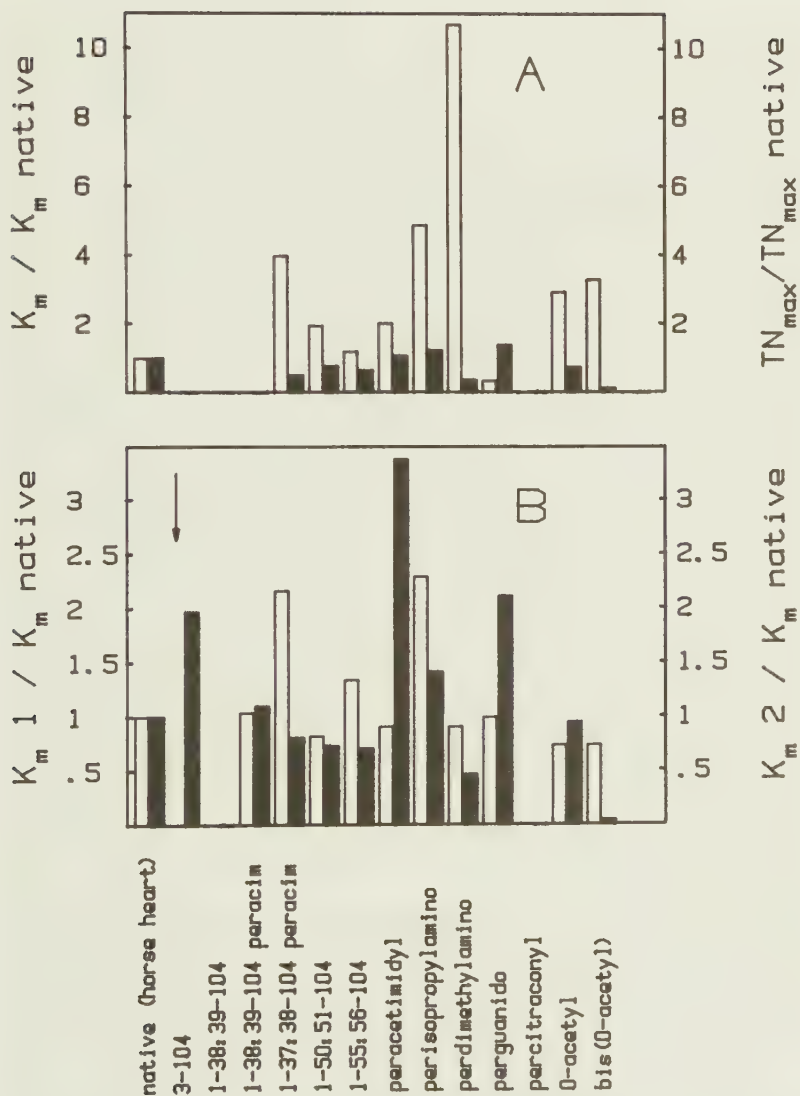


Figure 28: Summary of kinetics of modified cytochromes c

Panel A : High ionic strength : open bars K_m relative to native, cross hatched bars TN_{max} relative to native.

Panel B : Low ionic strength open bars K_{m1} , cross hatched bars K_{m2} .

chapter 4

DISCUSSION

4.1 *Molecular Weight of Oxidase*

Although it is relatively easy to obtain large amounts of oxidase, the purification methods have to be optimized carefully in order to get reproducible results. Due to the variability of phospholipid content, heme content, subunit content, and hydrodynamic radius, it is essential to thoroughly characterize each stock of oxidase. Moreover, comparison among kinetic and binding parameters should be performed with the same lot of oxidase. Most of the confusion about the molecular weight of oxidase is due to gel filtration artifacts and inappropriate correction for detergent content. One reason for artifacts is the ion exchanging property of most column materials at low ionic strengths. Other sources of error are reversed phase affinities of some gel materials for hydrophobic membrane enzymes. The oxidase sticks to the matrix, but it is remobilized by detergent micelles. Therefore, under certain conditions, an apparent molecular weight which is only slightly larger than the average micelle size is observed for the oxidase (see Figure 8 A, dotted line).

Cytochrome c oxidase was at least tetrameric when it was equilibrated in Tween-80 (Figure 7 B, solid line). Laurylmaltoside efficiently diminished the portion of oligomeric oxidase so that more than 60% was in the dimeric form (Figure 7 A, solid line). It was not possible to separate the oxidase in monomers and dimers, because the species were in a slow equilibrium and because the hydrodynamic radii of oxidase dimers and monomers were only slightly different (see page 41).

The only reliable method for the determination of the true molecular mass of oxidase is sedimentation equilibrium in solvents of different densities, all other methods being subject to systematical errors. The results from different laboratories using this method are unambiguous. The protein moiety of monomeric oxidase was found to have a molecular weight of 180 to 200 Kdaltons (page 29).

Triton X-100 and high pH treatment of oxidase clearly produced homogeneous monomeric oxidase (Figure 8 B, dotted line). The kinetic parameters of the oxidase prepared in this way were variable and the oxidase was more subject to ageing. But as the aggregation state of the oxidase did neither influence the binding of cytochrome c nor the kinetic parameters, it was not necessary to use pure monomeric oxidase for these studies.

4.2 *Binding and Kinetics of Oxidase*

If ligand binding is measured by the spectroscopic titration method, the difference extinction coefficient of bound minus free cytochrome c has to be determined from a double reciprocal plot of the titration curve. This determination influences the form of the curve in the Eadie Scatchard plot. In addition to that, errors are magnified in segments with a high

ratio of bound to free ligand. It is therefore not possible to use the shape of the Scatchard plot for the selection of a binding model.

Graphical representations of data together with theoretical curves can only give a subjective quantity of 'goodness of fit'. Selection of models has therefore been associated with qualities like a keen sense of smell or a touch of genius. Such decisions can lead to controversies and useless discussions, a fact often seen in biochemistry. Mathematical fits have the advantage that they provide an objective quantity of 'goodness of fit'. The only complication arises when models with different degrees of freedom have to be compared (see page 49).

A word of caution: Decisions can only be made based on good experimental data. No fitting routine is able to produce accurate results from a few inaccurate data points. It is essential that several sets of data measured under identical conditions are used for a thorough analysis.

The spectroscopic titration of oxidase was complicated by variability of oxidase absorption in the Soret region ($\pm 1\%$). When measured over prolonged periods of time the absorption increased or decreased in a random manner. An average of several stability measurements, however, was almost zero. Binding of cytochrome c to monomeric and dimeric oxidase was monophasic. The stoichiometry was always close to 1 heme c per heme a_{a3} . *P. denitrificans* oxidase had a lower affinity for cytochrome c, but otherwise it behaved identically.

Spectroscopic titrations and difference spectra have been measured in the fully oxidized state of both enzymes. In this case the affinity of the enzyme product complex was measured. A similar affinity was obtained from spectroscopic titrations of cyanide poisoned, fully reduced oxidase with ferrocycytochrome c.

The determination of equilibrium binding of cytochrome c to oxidase by the method of Hummel and Dryer resulted in one binding site with high affinity and another with low affinity per oxidase monomer. Several authors suggested that the low affinity 'binding site' is due to binding of cytochrome c to phospholipids. As cytochrome c, due to its strong dipole moment, binds to any polar group, this phenomenon has to be termed nonspecific binding.

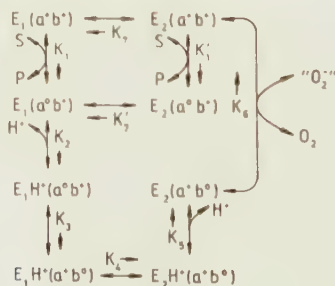
The main conclusion from the spectroscopic binding studies may be summarized as follows. Oxidase has only a single binding site for cytochrome c per heme a_{a3} , that is, per monomeric unit, irrespective of the aggregation state of the oxidase. There is no cooperativity between the cytochrome c binding sites in dimeric or oligomeric oxidase. This finding has important consequences for the mechanism of the steady-state reaction. Any mechanism based on low- and high-affinity binding sites (Ferguson-Miller et al., 1978), on negative cooperativity between a priori identical binding sites (Bolli et al., 1985a), or on active and regulatory sites (Errede et al., 1986; Speck et al., 1979; Sinjorgo et al., 1986) must be abandoned in favor of a mechanism explaining non-hyperbolic steady-state kinetics by two distinct conformational states of the oxidase. These states are a consequence of the electron transfer reaction, they are not preexisting or induced through nega-

tive cooperativity after the first cytochrome *c* has bound. The two conformational states may be related to vectorial proton pumping (Brzezinsky et al., 1986; Brzezinsky and Malmström, 1986).

Several authors have shown that cytochrome *c* oxidase pumps protons across the membrane. The stoichiometry of the number of protons per electron that is transferred to oxygen is controversial. One obligatory feature of an active proton pump is a redox linked conformational change, providing alternating access for the transported proton to the two sides of the membrane (Tanford, 1983). Coupling between exergonic electron transfer and endergonic H^+ pumping is achieved by energy linked conformational changes. Oxidized cytochrome *a* accepts an electron from cytochrome *c* at a reduction potential of 285 mV. When the electron is transferred to cytochrome a_3 , the potential of cytochrome *a* drops to 220 mV whereas the potential of cytochrome a_3 is raised from 285 to 350 mV. This strongly indicates that cytochrome *c* oxidase exists in two conformations. It is logical that these two conformations have different affinities for an electron and different kinetic constants for electron transfer. Such a thermodynamic linkage in an enzyme causes non-hyperbolic kinetics (Ainslie et al., 1972; Reynolds et al., 1985).

The results obtained at a variety of conditions in different detergents and at different pH clearly prove that biphasic kinetics is a general phenomenon of oxidase, whether the enzyme is dimeric or monomeric. Biphasic kinetics cannot be observed under all conditions. At ionic strengths higher than approximately 100 mM and at pH values below 6.8, steady-state activity of oxidase becomes linear. That means, high sodium ion concentration and/or high proton concentrations favor monophasic kinetics. The high and the low affinity phase merge to a single phase when one of the limiting conditions is passed. But the effect generated by sodium ions is not the same as the effect generated by protons. Whereas the maximal velocity reaches a maximum at 75 mM ionic strength and decreases when the ionic strength is further increased, no maximum can be observed when the proton concentration is increased (the pH is lowered).

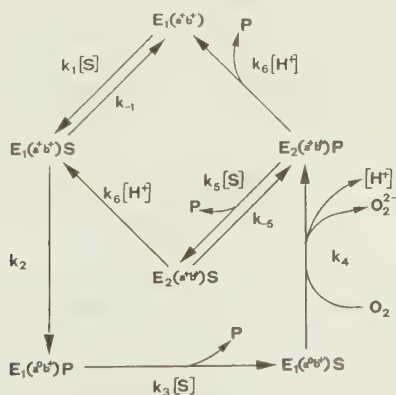
Brzezinsky and Malmström (1986) have proposed the following model for coupling the oxidation of ferrocytochrome *c* to H^+ pumping.



Model 1 : proposed by Brzezinsky and Malmström (1986)

Oxidase exists in two conformational states, E_1 and E_2 . For simplicity, only 2 redox centres a and b are considered. The equilibrium is in favor of the E_1 conformation when both redox centres are reduced and in favor of the E_2 conformation when redox center a is oxidized ($K_7' \gg K_7$, the arrows indicate the direction in which equilibrium constants K are defined). The essence of the model, however, is the different affinity of E_1 and E_2 for the substrate S , ferrocyanochrome c : $K_1 \gg K_1'$. The bottom loop of the scheme connects cytochrome c oxidation with H^+ pumping and O_2 reduction. The intramolecular electron transfer between redox centres a and b (bottom) is linked to a conformational change which, in turn, drives H^+ pumping. The circle is completed by the transfer of an electron to O_2 and reversal of the conformation from E_2 to E_1 . If reversal through equilibrium K_7' is preferred, as implied by Brzezinski and Malmström, there is a second low-activity phase of the steady-state reaction since $K_1' \ll K_1$. The rate equation for model 1 shows a quadratic dependence on the substrate concentration and is of the form of equation 5.

Model 1 does not account for monophasic kinetics at high ionic strength because K_1 decreases with increasing ionic strength, as experimentally verified, and merges in value with K_1' . Alternatively, the second phase is no longer visible under the experimental conditions because K_1 is getting too low. The model accounts for an increased reaction rate with increasing proton concentration, but it cannot explain why kinetics become monophasic at lower pH values. Therefore, following model which does explain monophasic kinetics at low pH as well as an ionic strength dependence of the maximal turnover number is proposed.



Model 2 : New model proposed for oxidase kinetics

In this model proton pumping is again coupled to electron transfer. As in Model 1, oxidase exists in 2 different conformations, interconnected by the absorption and the release of a proton, with E_2 being the deprotonated form. In accordance with published observations the H^+ stoichiometry is not fixed. In contrast to Model 1, a 2 electron transfer step is necessary before E_2 can return to E_1 , and a two electron-reduction is required for rapid electron transfer (Fabian et al., 1987; Scholes and Malmström, 1985). Biphasic steady-

state kinetics are explained as in Model 1 by K_1 ($k_1[S]/k_{-1}$) being much smaller than K_5 ($k_5[S]/k_{-5}$), that is, binding of ferrocycytochrome c to the E_1 conformation is favored. Again K_1 decreases at increasing ionic strength and merges in value with K_5 to exhibit monophasic kinetics. Lower pH speeds up the reaction by accelerating the conformational transition from E_2 to E_1 . Moreover, monophasic kinetics at low pH follow if $E_2(a^+b^+)P \rightleftharpoons E_1(a^+b^+)P \rightleftharpoons E_1(a^+b^+)S$. This must be the case if K_5 is pH independent. The rate equation of this model derived by the method of King and Altman, described in Clealand (1979) and Segel (1975), exhibits a third degree dependence on substrate concentration and a quadratic dependence on proton concentration for a H^+/e^+ stoichiometry of 0.5. Assuming a second proton binding site optionally transporting another proton could explain all stoichiometries of proton translocation between 0.5 and 1.

There is another difference to Model 1. Distinction between the high- and low-affinity phase of the reaction relies only on differences in the binding equilibria K_1 and K_5 and not on differences in binding and rate of electron transfer; K_1 and K_1' of Model 1 comprise two equilibria, binding of ferrocycytochrome c and electron transfer for the primary acceptor.

In the simplest case the rate constants for the conformational transition may be equal, (k_6). The composite rate constant k_4 must not be rate limiting if the kinetics are to be biphasic. The order of H^+ release and O_2 reduction is unimportant. The redox centres a and b are not specified.

An other interesting field of oxidase research is the localization of the binding site of cytochrome c on the oxidase. For this purpose the oxidase had to be split into subunits without denaturation of the oxidase. Spectroscopic titrations of an oxidase subunit (peptides I, II, and III) demonstrated that they were still able to bind cytochrome c (Figure 17 B).

Most interesting was the difference spectrum due to the binding of cytochrome c to the chymotrypsin treated oxidase (Figure 17 A). Chymotrypsin treated oxidase was still able to oxidized cytochrome c at a very low speed. Eadie Hofstee transformations were strictly monophasic.

4.3 The Nature of the Difference Spectra

Heme without a protein matrix is a system with many degrees of freedom. It can perform almost any electron transfer reaction at a very slow rate. The protein chain of cytochrome c restricts the freedom of the heme so that it can only transfer electrons to oxidase or related systems but at a very high speed. This restriction is visible from solvent perturbation spectra: Cytochrome c does respond to an altered environment in three ways only:

- Alcohols with one hydroxyl group open the heme crevice of cytochrome c. The difference spectrum is similar to the difference spectrum observed during the alkaline transition.
- Polyalcohols, sodium chloride, polyglutamic acid and phosvitin cause a difference spectrum with a similar shape, but a three to ten times smaller magnitude as observed for the binding of cytochrome c to oxidase.

- c) Perturbation of cytochrome c with glycine, an amino acid with a strong dipole moment, causes a difference spectrum with an opposite sign compared to the binding difference spectrum with oxidase. The same effect is observed when free cytochrome c forms dimers and oligomers at high concentration.

Observations b) and c) represent opposite changes of the electric field across heme c. Binding to oxidase and perturbation with the group b) agents reduce the electric field strength across heme c whereas glycine increases the field strength. The perturbations are observed in the absorption spectrum as well as in the MCD spectrum.

The MCD signal in the oxidized and in the reduced case is shifted to the red by 1 nm when cytochrome c is bound to the oxidase (Figure 19 A). The shape of the difference spectrum is a mirror image of the difference caused by the alkaline transition (Figure 21 A). The negative sign of the glycine perturbation spectrum of cytochrome c points out the influence of the electric field on the MCD spectrum. Based on the perturbation difference spectra and on the difference spectra of the alkaline transition, a less polar environment and less intensive electric field around the heme is proposed to be responsible for the MCD signal of oxidase bound cytochrome c. This is in accordance with the three dimensional structure of cytochrome c where about 90% of the heme are shielded from the solvent and 10% remain exposed. It seems that the iron geometry is not the primary source for the difference MCD spectrum particularly because the red shift has the same magnitude in the oxidized and in the reduced protein.

In conclusion : The MCD difference spectrum due to binding is not very specific. Similar difference spectra can be induced by solvent perturbations.

CD binding difference spectra and CD perturbation spectra have been measured under the same conditions as the absorption and MCD difference spectra. The CD difference spectra, however, are quite different :

- Solvent perturbations have much smaller effects on the CD spectra of cytochrome c than binding to oxidase. Hence, the oxidase alters the conformation of cytochrome c in order to prepare it for electron transfer.
- The CD spectrum due to binding of cytochrome c to oxidase is similar to the CD difference due to the alkaline transition.

The massive change in the environment of the heme c is pointed out by the large size of the CD-difference spectrum. It is straightforward to assume that these changes of conformation are not accidental. This observation is explained by the startling duality of cytochrome c:

Free cytochrome c is a good container for the electron whereas bound cytochrome c readily transfers and accepts electrons.

The changes in electron flow out of reduced cytochrome c when it is bound to cytochrome c oxidase are dramatic : Reduced cytochrome c in a buffer containing oxygen keeps its electron with a half life of at least one week ($k=10^{-6} \text{ s}^{-1}$). Reduced cytochrome

c bound to cytochrome c oxidase transfers its electron to heme a at a speed of $8 \times 10^6 \text{ s}^{-1}$ (Wilson et al., 1975; Andreasson, 1975; Van Buuren et al., 1974; Gibson et al., 1965). The electron transfer rate is increased by cytochrome c oxidase by a factor of 10^{13} . For conventional enzymes an increase in reaction rate by 10^9 is considered to be an upper level. This implies that oxidase alters the structure of cytochrome c, so that its surface changes from an ideal insulator to a very good conductor for electron flow. The extreme sensitivity of tunneling probability to the physical proximity and stereochemical matching of macromolecules can explain the 'unlocking mechanism' of cytochrome c.

Cytochrome c can be induced to transfer its electron to oxygen simply by increasing the pH (alkaline transition, see Introduction). The cause of this sudden autooxidizability is the disruption of one or two hydrogen bonds above and below the exposed heme edge, the Lys 13-Glu 90 and Lys 79 - Thr 47 hydrogen bonds. Astonishingly, the size of the CD differences at 414 nm that are observed during the rupture of these hydrogen bonds is similar to those observed during binding of cytochrome c to oxidase (Weber et al., 1987). The shape, however, is slightly different. The MCD difference spectra of the alkaline transition are much larger than the binding difference spectra and of opposite sign. This points out that the electronic structure is altered in a different way. In contrast to the binding, the heme c is rendered to a more polar environment during the alkaline transition.

Replacing the invariant phenylalanine 87 with either serine, tyrosine, or glycine by site directed mutagenesis resulted in a loss of the negative Cotton effect at 417 nm (Pielak et al., 1986). This Phe 87 corresponds to the Phe 82 of horse heart and tuna cytochrome c. It has been shown to be a surface residue with the phenyl ring parallel to the heme plane, and 5 Å apart from the methionine side of the heme. The difference spectrum modified ferricytochrome c minus native ferricytochrome c is closely similar to the difference spectrum of bound ferricytochrome c minus free ferricytochrome c. Not only is the shape of the difference spectra almost identical but also the size at 414 nm. This suggests that the movement of the Phe 82 out of the vicinity of the heme c is the major cause for the binding difference spectrum. Similarly, the difference spectrum modified ferrocycytochrome c minus native ferrocycytochrome c is closely similar to the difference spectrum of bound ferrocycytochrome c minus free ferrocycytochrome c (see Figure 20 B and spectra in Pielak et al., 1986). Thus, a movement of Phe 82 is the cause of the 414 nm CD difference signal irrespective of the electronic structure of the heme iron.

The proton magnetic resonance of several heme c methyl groups as well as a proton of the Cys 14 thioether bridge and a proton of Ile 75 or Ile 81 are shifted when cytochrome c binds to oxidase. The ^1H -NMR. pattern of bound cytochrome c is similar to the pattern of free cytochrome c modified at Lys 13. It is suggested that binding to oxidase causes a rupture of the salt bridge between Lys 13 and Glu 90 and a concomitant perturbation of the methionine ligand (Falk and Ångström, 1983).

Another line of evidence supporting this proposition is the observation of Satterlee et al. (1987). They have detected changes in the heme c electronic structure of bound cytochrome c by ^1H -NMR. The most prominent shifts have been observed for the heme 8

and 3 methyl resonances and for Met 80. They attribute the cause of these changes to a swing out movement of the Lys 13 after the rupture of the Lys 13 - Glu 90 hydrogen bond, and subsequent transmission of the effects from the cytochrome c surface to its heme vicinity.

The large movements of hydrophobic groups during the rearrangement of cytochrome c prior to the electron-transfer favor the tunneling mechanism (see Introduction). The Phe 82 is used to contribute to a hydrophobic channel, a series of stacked aromatic residues.

Based on the results the following binding model is proposed:

Cytochrome c is unlocked by the rupture of the Lys 13-Glu 90 hydrogen bond. These strongly polar residues are pulled toward the oxidase probably by newly formed hydrogen bonds. This pulls the loop 90 to 80 to the front and thus moves Phe 82 away from the heme plane. The upper right part of the cytochrome c, residues 1 to 16, might move to the front too, thereby tilting the heme by the Cys 14 and Cys 16 bonds and translating the movement to His 18.

Binding of cytochrome derivatives with a break in the polypeptide chain (called non-covalent cytochromes c in subsequent text) to oxidase results in significantly altered difference spectra compared to native cytochrome c (see Figure 24 A and B). This finding can be ascribed to partial alkaline denaturation of these derivatives since, based on the pK value for the alkaline transition, the difference spectra can be corrected for the contribution of the denaturation. These corrected spectra are closely similar to that of native cytochrome c (see Figure 24 C and D). A possible explanation for this result is that that oxidases are able to impose a "near native" conformation and electronic structure on the 'floppy' non-covalent derivatives.

Unlike with the above described derivatives, with acetimidylated non-covalent derivatives the corrected difference spectra are not similar to those of native cytochrome c. It seems that any modifying group on the surface of cytochrome c, particularly on the front side, reduces the match that is needed for the above described renaturation from the alkaline to the "near native" conformation. If the same modifying group is attached to cytochrome c without a non-covalent modification, almost no effect on the binding is observed (Figure 23 A).

The absorption difference spectra due to binding of cytochromes c to beef heart oxidase are maximal at 410 nm (Figure 18). The CD spectrum is maximal at 414 nm and the MCD difference spectrum is maximal at 408 nm (Figures 19 A and B). Even dif-

ference spectra due to binding of non-covalent cytochromes c to beef heart oxidase show the same maxima after correction (Figure 25). However, there are a few exceptions with maxima at different wavelengths.

Binding difference spectra of beef heart oxidase with perdimethyl and perisopropyl modified cytochrome c are smaller and at 406 nm (Figure 23). The same can be observed for *C. krusei* cytochrome c (not shown). Common to these cytochromes is their weaker affinity for the oxidase and their smaller difference extinction coefficient. Binding difference spectra of *P. denitrificans* oxidase with horse heart cytochrome c are of comparable size but the maximum is at 404 to 405 nm (Figure 18).

These observations imply that the absorption difference at 410 nm is indicative of the polarity of the environment of the heme c in the complex. *P. denitrificans* seems to bring other and probably less hydrophobic residues into close proximity of the heme c.

Bound chloramine-T-modified cytochromes c have optical spectra much more similar to native cytochrome c than free chloramine-T-modified cytochromes c (Figure 27 C). This applies to all the derivatives that have been measured (FII, FIII, and FIV). The shape of the CD difference spectra of the FII and FIII derivatives due to binding to oxidase is not similar to the CD difference spectra measured for native cytochrome c. A striking feature of these difference spectra is their large positive band at 414 nm. Again, it seems that this band is due to a movement of Phe 82 and that the altered electronic structure and conformation around the heme iron does not influence this movement.

The CD difference spectrum due to the binding of 3 - 104 cytochrome c to oxidase is maximal at 414 nm (Figure 26 D). At this wavelength the positive ellipticity is comparable to the binding of native cytochrome c. The maximum at 414 nm shows that Phe 82 still moves away from the heme vicinity. Below 408 nm however the CD difference becomes negative. With these features the CD difference spectrum is strikingly similar to the CD difference spectra of chloramine-T modified cytochromes c (Figure 27 D).

The MCD difference spectrum is roughly three times larger but still at the same position as the native difference spectrum. The 2 nm shift of the MCD absorption to the red when the 3 - 104 derivative is bound is indicative of a less polar environment in the bound 3 - 104 derivative. The large influence of residues 1 and 2 on the kinetic behavior (Figure 28 and Table 10) as well as on the difference spectra can be explained by a role of these residues in the formation of the hydrophobic channel to the redox partner.

The 3 - 104 cytochrome c has a blue shifted absorption maximum. It is quite similar to the spectrum of alkaline cytochrome c, but the 3 - 104 is hardly autooxidable.

Most interesting is the lack of a high affinity kinetic phase at low ionic strength for this derivative. The affinity however is only two to three times weaker than with native cytochrome c. This is in accordance with the slightly different electric dipole vector. The results from the steady-state kinetics point to an altered unlocking mechanism or to a less efficient hydrophobic channel.

4.4 Outlook

The structural rearrangements that occur during the binding of cytochrome c to oxidase are highly specific. It is not possible to obtain similar structural rearrangements when non-heme ligands are bound to cytochrome c. Therefore, the only possibility to acquire more information is by variation of either binding partner. For several reasons cytochrome c peroxidase is a suitable substitute for oxidase :

- The absorption spectrum of a complex of cytochrome c peroxidase with cytochrome c is slightly different from the sum of the spectra of the components.
- The difference saturates at a ratio of 1 heme c per heme of peroxidase.
- The three dimensional structure of peroxidase is known, which would help in the modeling of the electron transfer complex.

Features of the optical spectra can be further investigated by using bacterial oxidases, bc₁ complex, cytochrome b₅, photosynthetic reaction centres, and other redox partners of cytochrome c.

Horse heart cytochrome c can be substituted by cytochrome c from other species, by cytochrome c₂ and by cytochromes with a different chirality around the heme iron (e.g. *P. aeruginosa*).

Chemically modified cytochromes can be used to acquire more information on electron transfer. The 3 - 104 derivative, where the lack of the conserved Asp 2 induces large changes in kinetics as well as in the different spectra, is very promising.

More information has to be collected at different wavelengths (e.g. aromatic region, 695 nm band) and with different methods (e.g. ¹H-NMR, fluorescence). Measuring difference spectra in the aromatic region could specifically detect the altered environment of phenylalanines. The 695-nm band of cytochrome c can be used to check the integrity of the heme-iron-methionine sulfur bond in bound cytochrome c. Results from two dimensional NMR could help to locate the structural and electronic rearrangements more precisely.

The influence of single residues on binding and kinetics can be tested by site-specific mutagenesis of cytochrome c (Liang et al., 1987). From the results measured this way, the structure of the hydrophobic channels could be traced.

Another field of interest is the conformational change of oxidase during the catalytic cycle. Due to the complexity of the oxidase, a better insight into the principle of this change might need a lot of time. One possible advance could be achieved by investigating oxidase that lacks certain subunits, or by measuring binding of cytochrome c to oxidase sub-species.

chapter 5

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PART 2

**Automatic Determination
of
Binding and Kinetic Constants**

Summary

Measuring binding and kinetic constants of enzymes is a central problem in biochemistry. Up to now the determination of these constants was exceedingly time consuming. For this reason a computer controlled binding titrator and an automatical assay mixing robot were developed.

The spectroscopic binding titration uses minor spectral differences induced in both enzyme and ligand to determine the amount of bound ligand. There is no need to separate the complex from free ligand. Differences in the range of 1 - 2% of the total absorbance are sufficient to distinguish the binding signal from the absorption of enzyme and ligand. This superior sensitivity could be achieved by highly accurate titrations and mathematical elimination of systematical errors. For this purpose a computer controlled titrator was designed. It is able to reproduce series of titrations with an accuracy of 0.1 μ l for a total volume of 500 μ l. A major part of the systematical errors is due to stray light. This can be corrected mathematically. Other errors are corrected by blank measurements.

Assays are mixed from stock solutions of substrate, enzyme, and buffer by means of the Assayomate. This instrument was designed to mix assays of varying substrate concentration (up to a factor of 500) as necessary for the determination of kinetic constants.

The MICHKIN software controls the Assayomate and calculates the kinetic constants by linear regression to a set of velocity data measured at different substrate concentrations. Comparison of kinetic constants determined with the Assayomate with published values proved the reliability of this instrument.

The reproducibility of automatically determined kinetic constants is much better than with manual methods. In addition, use of the instrument saves a lot of working time.

The MICHFIT software handles the files generated by the spectroscopic titration and by the MICHKIN software. The major task of the MICHFIT software is direct curve fitting to complex binding and kinetic models and graphical presentation of results. All the fits and the majority of the plots in this thesis have been performed with this software.

Zusammenfassung

Die Bestimmung von Bindung und von kinetischen Konstanten ist sehr wichtig in der Biochemie. Bis jetzt war die Bestimmung dieser Konstanten sehr zeitraubend. Aus diesem Grund wurde ein computergesteuerter Titrator und ein Assay- Misch-Roboter entwickelt.

Die spektroskopische Titration benützt kleinste Unterschiede, die beim Binden von Ligand und Enzym entstehen, um die Menge des gebundenen Liganden zu bestimmen. Daher muss der Komplex nicht vom freien Liganden getrennt werden. Unterschiede im Bereich von 1 bis 2% der gesamten Absorption des Enzyms und des Liganden reichen aus, um das Bindungssignal zu messen. Diese hervorragende Empfindlichkeit konnte durch sehr genaue Titrations und durch Korrektur von systematischen Fehlern erreicht werden. Zu diesem Zweck wurde ein computergesteuerter Titrator entwickelt. Dieser wiederholt Titrations mit einer Genauigkeit von 0.1 μl auf 500 μl . Ferner wurde ein Programm geschrieben, das die systematischen Fehler mathematisch korrigiert.

Der Assayomat mischt Assays aus Vorratslösungen von Substrat, Enzym und Puffer. Dieses Instrument wurde entwickelt um Assays mit verschiedener Substratkonzentration (um einen Faktor 500 verschieden) zu mischen. Dies ist nötig, um kinetische Konstanten automatisch zu bestimmen.

Die MIC²KIN Software steuert den Assayomaten und berechnet die kinetischen Konstanten mittels einer linearen Regression aus den Enzymaktivitäten bei verschiedener Substratkonzentration. Der Vergleich der kinetischen Konstanten, die mit dem Assayomat bestimmt wurden, mit veröffentlichten Werten, beweist die Verlässlichkeit dieses Instruments. Die Wiederholbarkeit von automatisch bestimmten kinetischen Konstanten ist viel besser als bei manuellen Methoden. Zudem spart der Einsatz dieses Instruments viel Arbeitszeit.

Die MICHFIT Software verarbeitet die Daten, die vom BIRATE, und vom MICHKIN Programm abgespeichert wurden. Die Hauptaufgabe der MICHFIT Software ist nichtlineare Regression (Curve Fit) und graphische Darstellung von experimentellen Daten. Alle Regressionen und nahezu alle Figuren in dieser Arbeit wurden mit dieser Software ausgeführt.

chapter 6

INTRODUCTION

In biochemistry most of the experimental routine is still done by hand, even such tedious operations as enzyme assays, binding assays, determination of inhibition constants, redox titrations, and pH titrations. This is due partly to the complexity of many biochemical manipulations, and partly to the small number of prospective users for most of the automatic devices. In other sciences, the degree of automation is much higher.

Recently, biochemical, immunological, and genetical research has found wider application in industry. The marriage of these sciences with engineering for the purpose of industrial production of goods is now called **biotechnology**.

In the past three years several instruments that increase the efficiency of biotechnological research have reached the market. For example, protein sequencers (instruments used to analyze the composition of proteins) and DNA synthesizers (instruments that can automatically build customized DNA chains) have enabled scientists to quickly identify and mass produce genes of interest. Because virtually all products of genes are proteins, any biotechnological laboratory must have equipment suitable for handling and analyzing these materials.

With genetic engineering it will be possible to tailor enzymes * for the specific need of production or research. But for the selection of the enzyme best suited for a desired purpose, a large number of candidate enzymes have to be tested. In production, the most suitable enzyme is often the enzyme which can produce most of a desired product without contamination of by-products. The rate of production is related to the **steady-state activity** of an enzyme. It depends on the **affinity** of the enzyme for the substrate, the amount of enzyme, and the steady-state activity of a single enzyme molecule. If substrate concentrations are very high, the enzyme is saturated, working at the **maximal velocity** ($V_{\max}$). At low substrate concentrations, the apparent velocity depends on the ability of the enzyme to bind its substrate. This ability is expressed by the **Michaelis constant** (K_m) and the **dissociation constant** (K_d). Another parameter describing the quality of an enzyme is the **specificity** of the enzyme for a substrate. The specificity is determined by comparing the ratio $V_{\max}/K_m$ of different substrates.

The activity of many enzymes depends on the concentrations of inhibitors or activators. The effect of these substances is characterized by the **inhibition constant** (K_i) and the **activation constant** (K_a). Knowledge of these constants is especially important for drug research. The correct pharmacological dose of a certain drug should inhibit enzymes of bacteria or virus (e.g. penicillinase, reverse transcriptase) without affecting

* An enzyme is a protein that enhances the speed of a chemical reaction by lowering the energy of activation of the reaction. It is specific for one or a small number of substrates.

vital functions of the mammalian metabolism.

For the reasons just stated, the optimization procedure depends crucially on the exact determination of the kinetic and binding parameters of an enzyme.

Until now screening procedures for enzymes had to be restricted to the determination of an approximate **maximal velocity**. The determination of the exact parameters was omitted simply because of the large amount of labour time needed for the measurements. A determination of the **Michaelis constant** was performed with a small number of selected enzymes only. As many bacteria tend to alter their genes very rapidly, it is essential to monitor constantly during production the parameters of the expressed enzymes.

With the systems presented here it is possible to accurately measure several kinetic parameters of a large number of enzymes at low cost.

The MICHKIN software in conjunction with the Assayomate is capable of measuring the **MICHAELIS CONSTANT** and **MAXIMAL VELOCITY** of an enzyme fully automatically in less than 10 minutes. The BITRATE software measures the **dissociation constant** of an enzyme substrate complex within half an hour.

The task described in this part of the thesis was the design of a binding and a kinetic data acquisition-system. Both systems have been designed by a user with the intention to meet optimally the demands of other users. The entire design process was constantly oriented on experience collected during tests and first applications. Therefore, it was possible to shorten the duration of design, test, and redesign phases considerably. The binding and kinetic data acquisition has been automated from the beginning, they do not just consist of a computer added to an existing manual process. Moreover, both processes work best if no errors are introduced by manual interference.

In addition to the data-acquisition system, a data processing program was developed under the MS-DOS operating-system. It is used to plot measured data together with simulated curves generated from the results of a direct curve fit subroutine. This was done with the intention to handle the data in an optimally tuned environment without the need to convert data from one format to another.

The application of the two automatical devices demonstrated that it is possible to save a lot of working time in biochemistry.

chapter 7

BINDING TITRATOR

7.1 Introduction

All enzymes are large protein molecules, but only a small fraction of the enzyme is actually involved in catalysis. The rest of the enzyme is responsible for the recognition of substrates, inhibitors and activators. Enzymes enhance reaction rates by lowering the energy of activation for the conversion of substrate into product. The active site aligns the orbitals of the substrate and the catalytic groups optimally to enter the transition - state. Certain bonds of the substrate are distorted upon binding to the enzyme, this is called '**rack mechanism**' or '**induced fit**'. The altered environment of groups on the surface of the enzyme and the induced fit can change physical properties of both, the enzyme and the ligand.

7.1.1 Measuring Binding of Ligands to Proteins

Measuring noncovalent binding between molecules is a major problem in biochemistry. For this reason, several methods have been developed:

- a) **Method of Hummel and Dryer** : The enzyme is applied to a gel filtration column that has been equilibrated with ligand. The enzyme binds the ligand and therefore the complex with the bound ligand elutes at a position different from the free ligand. The amount of bound ligand has to be determined at several different ligand concentrations. This is a lengthy procedure that needs a lot of enzyme and is subject to large errors.
- b) **Radio immuno assay (RIA)** : If a radioactive ligand is available, the affinity can be measured by displacement of the radioactive ligand by different amounts of 'cold' ligand. The free ligand has to be separated from the complex and measured by liquid scintillation counting. The separation step is sometimes impossible or introduces a large error.
- c) **Ultrafiltration** : The enzyme and the ligand are applied to the compartment A separated by a membrane permeable only to the free ligand from compartment B. The free ligand can be measured in compartment B after the system has reached equilibrium, this takes several hours. Again, for the determination of a dissociation constant, measurements have to be repeated at different enzyme to ligand ratios.
- d) **Fluorescence quenching** : The fluorescence intensity of certain groups on the surface of the enzyme's active site or of the ligand are sensitive to their surrounding. This can be used to quantitate the amount of bound ligand. The disadvantage of this method is the need of very pure substances, as a lot of impurities disturb fluorescence.

- e) Absorption difference, four cell method : There is often a difference of the light absorption between the complex and the sum of the absorptions of free enzyme and free ligand. The difference is proportionate to the amount of bound ligand. This method works well if the induced absorption difference is large.

All these methods suffer from severe drawbacks : For methods a) to c) the free ligand has to be separated from the bound species, this is a lengthy procedure that introduces large errors in the determination of the ratio of bound ligand to free ligand. As not all enzymes exhibit fluorescence, method d) is limited to special cases. The absorption difference method (e) is also limited, although less than the fluorescence quenching method. Tandem cuvettes are used to measure the difference spectrum in method (e).

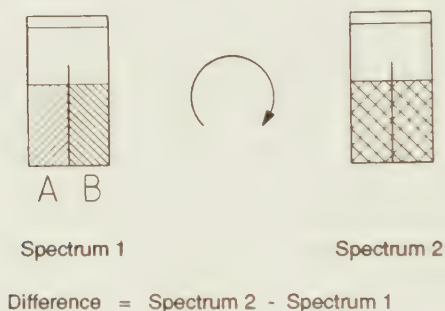


Figure 29: Difference spectrum with tandem cuvette

This method can be applied when the difference is large compared to the absorption of ligand and enzyme. If the difference is small, the errors introduced by tilting the cuvette, by pipetting and by different pathlengths of the two compartments are larger than the difference itself. Another drawback of this method is the large amount of enzyme needed; each point of a titration curve has to be mixed with new enzyme. Unstable enzymes alter their properties during the long measuring times needed to measure a titration curve. The binding titrator was developed in order to overcome these drawbacks:

7.1.2 Aims

The aim of the work presented here was the design and construction of an automatical binding titrator. It should make use of spectroscopic changes in the ligand induced by the enzyme, without the need to separate the complex from the free ligand.

Up to the present time small spectroscopic differences that are not accompanied by a large peak-shift have been impossible to measure quantitatively. Therefore, an automatical procedure would be a major advance in the field if it could increase the accuracy and sensitivity of binding titrations to the order of less than two percent total absorption difference. This aim has come into reach with the new generation of spectrophotometers which use an array of photodiodes to measure light intensity. A major advantage of diode array spectrophotometers is their superior stability and sensitivity as well as their faster data acquisition rate.

As accurate automatical titrators were not commercially available, a computer controlled titrator should be designed and constructed as a part of this thesis.

The design included the selection of an appropriate hardware :

- precise volume additions under computer control
- repetitive additions
- good mixing of the content of the cuvette
- stable temperature
- stable photometer with remote control

and the necessary software for the integration of all functions :

- set up of photometer
- reading and storing of parameter files
- control of titrator
- printing, plotting and storage of data
- stray light correction
- measuring of I_0 and I_s
- data transfer to host computer

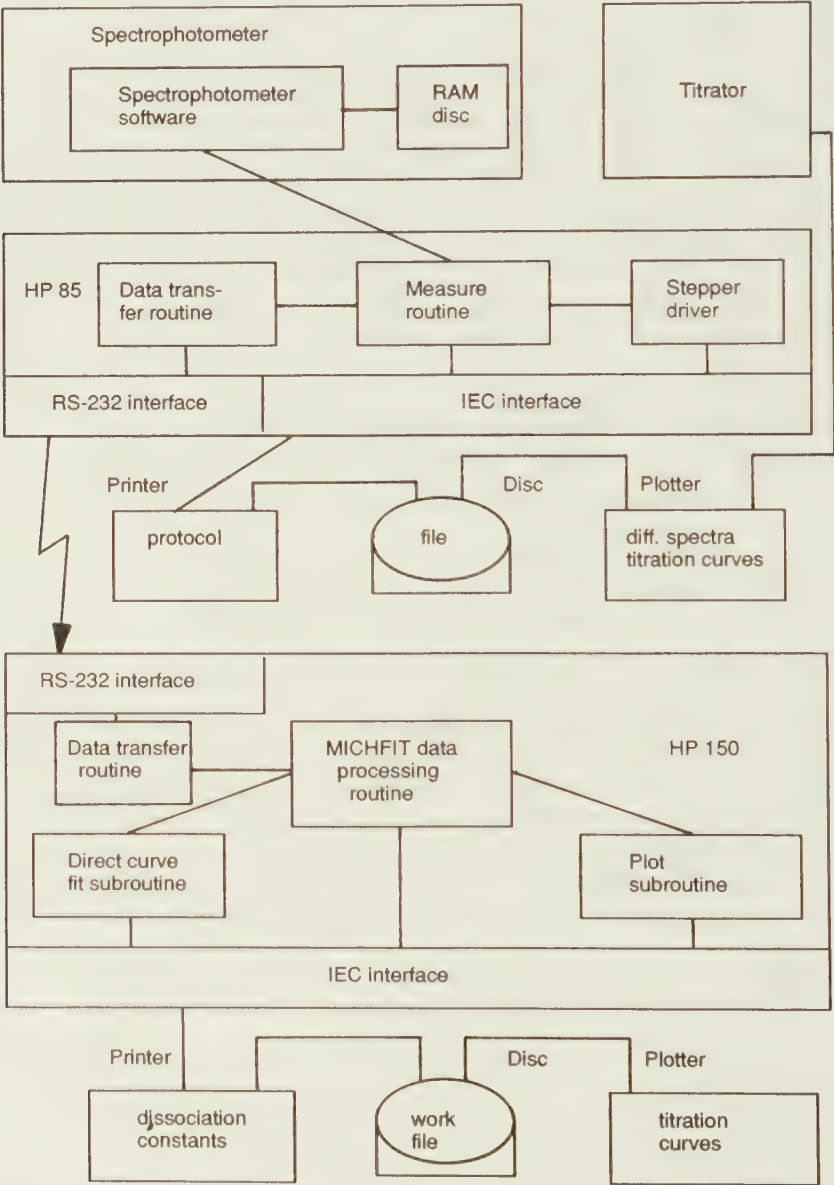


Figure 30: System concept of BITRATE software

7.2 System Concept

A complete system for the automatic determination of kinetic constants with spectroscopic titrations is composed of four major parts : (i) the spectrophotometer, (ii) the titrator, (iii) the computer controlling the cooperation of photometer and titrator, and (iv) a host computer for processing the data (Figure 30).

The spectrophotometer software measures spectra, stores them temporarily on a RAM disc, and transfers them to the HP 85 computer. A printer, a plotter, a disc drive and the titrator are connected to the HP 85 personal computer by means of an IEC bus. The BITRATE software controls the photometer, with ROM based system software and the titrator with a RAM based stepper motor driver. The BITRATE software is responsible for the acquisition and storage of an array of spectra, for the correction of systematical errors and for the calculation of a titration curve. After the data acquisition, the files are read from the disc and transferred to a host computer (HP 150) by means of an RS-232 interface.

The MICHFIT software on the HP 150 is able to evaluate dissociation constants from titration curves with a direct curve fit routine (Figure 30, lower box). Simulated curves from the results of fits together with experimental data are graphically presented by the plot subroutine. Experimental data and simulated curves are stored in workfiles or in plot files on disc. A printer, a plotter, a hard disc and a floppy disc drive are connected to the HP 150 with an IEC interface.

7.3 Structure of the Hardware

Four peripheral devices are connected to the HP 85 : (i) the photometer, (ii) a disc drive, (iii) a plotter, and (iv) the stepper motor controller (Figure 31). Disc drive and plotter are connected to the HP 85 by an IEC interface (see HP 8451 Owner's Manual). The stepper motor controller could be driven using the same IEC bus (see Titrator Subsection and Appendix E). It uses an IEC interface, a power supply, and the logical circuits that move the stepper motor (Figure 31, SM). The stepper motor drives a micrometer screw that moves a high precision syringe (A), so that very accurate aliquots (amounts) of ligand (L) can be added to the enzyme (E) in the cuvette (C). A magnetic stirring rod (R) driven by the magnet (M) constantly mixes the carefully thermostated content of the cuvette.

For a measurement, the shutter (S) is opened and the light (I_0) reaches the cuvette. A portion of the light is absorbed by the enzyme and ligand in the cuvette. Thus, the amount (I) of light that emerges from the cuvette is smaller than the light intensity (I_0). The light (I) is split into a spectrum (the portions of light of different color or wavelength are separated) by the prism (P) and the amount of light is measured at 321 different wavelengths with an array of photodiodes (D-A). The photodiode circuits furnish electrical voltages that are proportionate to the amount of light that has reached the light sensitive sur-

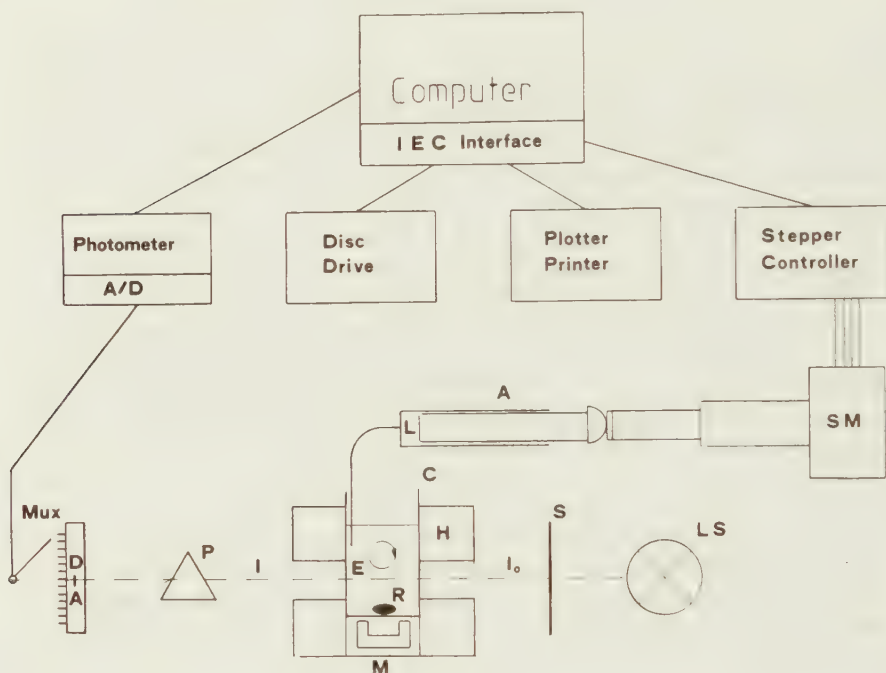


Figure 31: Survey of binding titrator hardware

A = Agla syringe A/D = analog to digital converter C = cuvette D-A = diode array E = enzyme H = thermostatable cell holder I_0 = light intensity of light source I = light intensity after cuvette L = ligand LS = light source M = magnetic stirrer Mux = multiplexer P = prism R = magnetic stirring rod S = shutter SM = stepper motor

faces. The voltages are amplified by 321 pre-amplifiers. The content of the pre-amplifiers of the 321 photodiodes is read out and digitalized sequentially by a multiplexer (Mux) and an analog to digital converter (A/D). This process is repeated 50 times with periods of 100 milliseconds. After 5 seconds the termination of the measurement is signalled to the controlling computer. If the computer is not busy, the spectrum is transferred and corrected for stray light. If it is found stable enough, it is displayed on the screen, stored on disc, and the next aliquot of ligand is added.

Spectrum scan time	0.1 second
Wavelength range	190 to 820 nm
Wavelength accuracy	+2 nm
Wavelength reproducibility	+0.05 nm
Spectral bandwidth	2 nm
Full dynamic range	0.0022 to 3.3 AU
Photometric accuracy	+0.009 AU
Baseline flatness	< 0.0013 AU
Noise at 0 AU (500 nm)	< 0.0002 AU
Stability at 340 nm	< 0.0028 AU/hr
	< 0.0010 AU/min
Stray light at 340 nm	< 0.05 %
HP 89050 A	Advanced techniques module
HP 92903 A	16 kBytes memory module
HP 82909 A	128 kBytes memory module
HP 82936 A	ROM drawer
HP 89057 A	Alphanumeric keyboard kit
HP 89054 A	Thermostatable cell holder
HP 89055 A	Cell stirring module
HP 00085-15007	Assembly language programming ROM

Table 11: Performance and accessories of HP 8451 spectrophotometer

7.3.1 Photometer

The HP 8451 Diode-array spectrophotometer is a single beam, microprocessor controlled, UV-Vis spectrophotometer which operates at high speed. Absorbance spectra over the full 190 to 820 nm wavelength range can be obtained in 0.1 seconds with a 0.7 second repetition rate. Values at up to 25 wavelengths can be obtained every 0.1 second. The radiation source is a deuterium lamp which emits radiation over the range 190 to 820 nm.

Central to the operation of the HP 8451 are two 8-bit microcomputers, the Z-80 and the HP 85A.

The Z-80 microcomputer controls the internal hardware (lamp, shutter, preamplifier and 14-bit analog to digital converter) and performs measurements. The operating system of the spectrophotometer is fixed in ROM's (24 kBytes). In addition to that it uses 16 kBytes RAM.

The HP 85A personal computer incorporates 56 kBytes ROM and 32 kBytes RAM. In addition to the operating system (32 kBytes) a BASIC interpreter is stored in the ROM's. The HP 85A microcomputer handles data, controls peripherals and functions as an interface between the user and the spectrophotometer. The HP 85 is equipped with a built-in CRT, a printer/plotter, and an alphanumeric keyboard.

Important features of the HP 8451 include :

1. Single beam elliptical optics and a holographic grating.
2. Diode array detector consisting of 328 photodiodes.
3. Processor aided measurements yielding direct answers in absorbance, transmittance or other user defined function.
4. Eight I/O ports for plug-in ROM and RAM memory, and interfaces, allowing communication with plotters, printers, disc drives, computers, and other peripherals.
5. Program storage : 16 kBytes RAM standard, additional 16 kBytes.
6. 128 kBytes RAM for storage of spectra

7.3.2 Titrator

The HP 8451 uses an IEC bus to transfer data to disc drive, printer and plotter. The same bus line can be used to control up to 15 peripheral devices. In addition to the 8 data lines, there are several control lines one of which, the REN line, is not used in this configuration (see HP Series 80 IO Manual). For this reason, the REN line was used to activate (enable) the stepper motor controller. Data line 2 (IO 2) controls the direction (D) of the stepper motor. A step is executed when a short pulse (S_1) is sent on data line 1 (see Appendix E).

The Agla MS 01 micrometer syringe outfit was developed for use in experimental techniques requiring the accurate measurement of very small volumes of fluid. It consists of a specially made and calibrated all-glass syringe, attached by a rigid holder to a micrometer screw gauge which operates the plunger.

Since the manual titration of large numbers of difference spectra was subject to errors, an automatic drive for the micrometer screw was designed. For this purpose, a stepper motor drive extruding $0.1 \mu\text{l}$ per pulse was designed.

The precision and reproducibility of titrations is best when the Agla syringe is kept at a constant temperature. It is therefore important to avoid touching the glass cylinder of the syringe or the micrometer during titrations. Moreover, no physical force must be exerted on any part of the drive. For these reasons it was necessary to develop a support for the syringe-stepper motor unit that could be moved without force (fully counterbalanced) and without touching the drive.

7.4 Structure of the Software

The software is separated in three parts, the system software, the driver software for the stepper motor, and the user software.

The system software controlling the interfaces, the peripheral devices (Display, Printer, Photometer), the organization of the processor and of the memory was supplied by Hewlett Packard.

The stepper motor is driven from the main processor via the IEC interface. For this purpose an extension to the system software was written.

The user software performs a dialogue between the user, the photometer, the titrator and the peripheral devices.

The computer in charge of controlling the overall system is a HP-85. It uses a specific ROM based operating system.

7.4.1 System Programs

The core of the system programs is built in ROM of the HP 8451, it is supplied directly with the computer. Additional functions can be added in additional ROM's. The configuration of the system was :

- Spectrophotometer software
- I/O- Rom
- Mass storage ROM
- Plotter/Printer ROM
- Assembler ROM

7.4.2 Stepper Motor Driver

The stepper motor is controlled by a RAM based extension to the system programs. This extension implements the BASIC command word:

MOTOR (Motor #),(direction),(1/speed),(# pulses)

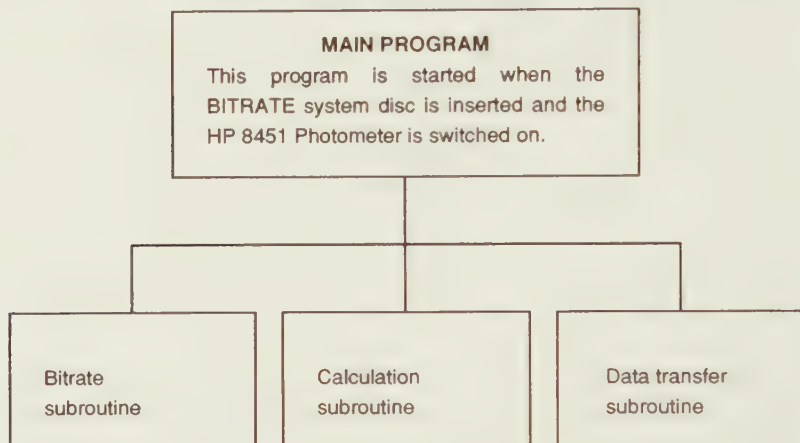
valid range : 1 0=forward 0-1000 0-5000

It has to be loaded into memory after the computer has been switched on. (This is done automatically by the main program of the user software). The command word 'MOTOR' expects four integers, separated by three commas. The first integer is used to select the bit that will be pulsed (1-7). The second integer is used to set all the other bits (0-255). The third integer specifies the number of wait cycles after each pulse. The fourth number is used to set the number of pulses that are executed (0-5000).

The stepper motor driver starts at a slow speed (more wait cycles), then it accelerates to the maximal speed. At the end of the move the speed is again decelerated.

7.4.3 The User Software

The user software is split into a main program and three subroutines :



Functions of programs

a) Main program ('Autost')

- Starts BITRATE software
- Initializes COMMON data block
- Initializes data discs
- Implements main menu (1st level menu)
- Activates subroutines
- Reads files containing measurement parameters
- Stores measurement parameters in files
- Displays disc directories
- Purges files
- Implements measurement parameter menu (2nd level menu) for change of (e.g. λ -ranges / duration / concentrations)

b) Bitrate subroutine

- Implements bitrate menu (2nd level menu)
- Measures difference spectra
- Measures light intensity (I_0) and stray light (I_s)
- Alters number of stored full spectra
- Controls the syringe (fill / empty / suck in / titrate / wash)
- Implements Syringe control menu (3rd level menu)

b) Calculate subroutine

- Implements calculate menu (2nd level menu)
- Calculates and plots titration curves and difference spectra from data that is already stored on RAM disc of photometer

d) Data transfer subroutine

- Implements Data transfer menu (2nd level menu)
- Transfers files to host computer via serial interface
- Implements Copy menu (3rd level menu)
- Copies files
- Edits files

7.4.3.1 Main Program

The main program of the BISTRATE software is started automatically when the system disc has been inserted before the HP 8451 photometer is switched on. Starting the BISTRATE software without switching on the photometer is done by typing LOAD "Autost" (+ end line) and pressing the 'RUN' key thereafter.

Approximately 20 seconds after the 'Autost' program has been started, a first screen is displayed. At this point the name of the data disc volume (6 characters) and the data have to be entered. The name of the volume is used to identify the data disc among several discs that are inserted in different drives connected to the HP 8451 photometer. The date is used in the same format as it was entered for print out and storage.

If this program is called for the first time, all system variables are initialized. A set of default values is loaded into the measurement parameters. Then the extension to the system programs is loaded into memory. After the completion of these tasks, the initiation flag (Z8) is set, this prevents another initiation when the program is called again. After the initiation has been finished the main menu is displayed.

As in all subsequent menus, 12 lines of information are displayed. At the bottom another two lines containing 8 short labels are displayed. These labels correspond to 8 functions that are accessible by the 8 softkeys * at the HP 85 computer.

Measurement parameter files (see Appendix C) are read from the system disc with the function 'Read Pa' and written to the system disc with the function 'Sto Pa'. They contain all information that is necessary to perform a binding titration. The contents of the measurement parameters can be changed by means of the measurement parameter menu. This second level menu can be accessed from the main menu with the softkey 'Meas Pa'. As in all other menus 12 lines of information together with 8 short labels are

* Definition of a softkey : A softkey is a key whose function is defined and redefined by the software. The functions that are assigned to the softkeys are displayed by the 'key label' key.

```

**   Main Program   Main Menu   **
*****
**   BITRATE  software for the   **
**   automatical titration of    **
**   dissociation constants      **
**   B. Michel   Aug. 17. 1987   **
*****

      Please select a softkey:
      (all other keys stop the program)

```

Direct	Plot PG	Sto Pa	
Bitrate	Dacom	Read Pa	Meas Pa

Figure 32: Main menu

The functions of the softkeys are :

- Softkey 1 'Bitrate' calls the measure subroutine.
 Softkey 2 'Dacom' calls the data transfer subroutine.
 Softkey 3 'Read Pa' activates a routine that prompts for a filename and reads a measurement parameter file.
 Softkey 4 'Meas Pa' selects the measurement parameter menu.
 Softkey 5 'Direct' shows directories of discs.
 Softkey 6 'Plot' calls plot program.
 Softkey 7 'Sto Pa' stores the measurement parameters in a file.

displayed. In this menu however, 8 softkeys are not sufficient to perform all functions. For this reason softkey #4 is used to select either a first or a second set of softkeys.

The function 'Durat.' is used to edit the duration of pausing and the duration of the measurement. It is important to wait after the addition of ligand until the content of the cuvette has become completely homogeneous (Figure 35 A). The duration of the measurement should be selected considering two opposite effects: Signal to noise and baseline shift. The signal to noise can be improved when the duration of the measurement is increased. In contrast, the baseline shift is minimal when the measurement is very short. As a rule, the best results are achieved when the duration is selected between 5 and 20 seconds.

The same criterion applies to the number of points of a titration curve (function 'Points'). If enzyme and ligand are optically stable, a titration curve of up to 80 data points can be performed. In most cases, however, a better accuracy is achieved when between 10 and 40 titrations steps are selected.

The noise parameter is used to reject inaccurate measurements if the standard deviation of many repetitive measurements is too high. There are two sources of inaccuracy that can be recognized :

Mixing time	15 s	Measure	12 s
Points	40	Max. Noise	0.005
λ -range titration	390 to 450 nm		
λ -range full spek.	390 to 630 nm		
λ -range normalize	610 to 630 nm		
Delta X :	6	Delta Xo :	10
Delta Y :	.005	Delta Yo :	.005
Cytochrome c oxidase	10 μ M (K)		
Cytochrome c	50 μ M (S)		
Initial vol.	2000 μ l	Mode	Int. Ref.
Monitor wavelength	410 nm		
Titration curves :	410 and 416 nm		

Durat.	Points	Noise	λ -Titr.
Exit	λ -Spek	λ -Norm	Page 2

Second set of softkeys :

Name	Conc.	λ -Curve	Mode
Volume	Full Sp	Conv.	Page 1

Figure 33: Measurement parameter menu

- short peaks due to lamp instability, electronic noise, air bubbles in the cuvette, or dust particles.
- slow shifts due to a slightly inhomogeneous mixture at the beginning of the measurement.

The total measuring time is split into two submeasurements each lasting for half of the total measuring time. The HP 8451 provides a standard deviation for both of these measurements (see Photometer Section). Short peaks are recognized if one of the standard deviations is too high. Slow shifts are recognized when the two measurements have not provided the same result. Depending on the type of error one or both measurements are repeated. The value of the noise parameter should be selected so that it is approximately 10 times smaller than the expected difference signal, but not lower than 0.001 AU. The next three functions are used to specify the total wavelength range (λ -Spek), the wavelength range for difference titrations (λ -Titr.), and the wavelength range for normalizing the results (λ -Norm).

The total wavelength range is limited by the storage capacity of the computer (max. 300 nm, 150 data points). Moreover, the wavelength range must not be selected for regions where the buffer and/or the enzyme absorption exceeds 2 AU. The normalizing range is used to pull down shifted spectra due to lamp fluctuations (see Test Section). It has to be selected in regions where neither enzyme nor ligand absorb light. In addition to that it has to be selected within the 300 nm total wavelength range.

There are two reason why the difference titration range should be kept narrow. The first is the slow speed of the stray light correction routine, the second is the limited amount of disc space (180 KBytes). A series of 40 difference spectra with a size of 100 nm needs 8 kBytes of storage space.

In addition to the block of difference spectra a series of spectra can be stored and transmitted (see Appendix C). The number of these spectra is entered with the 'Full Sp' function. The function 'Name' and 'Conc.' are used to enter the name of enzyme and ligand and the concentration of the respective stock solutions in μM . Together with the ' λ -Spek' and ' λ -Titr.' the X-Axis of the plots (ΔX and ΔX_0) have to be entered (see Sample Result).

The wavelength of the plotted titration curves are selected with the ' λ -Curve' function. The first wavelength is used to monitor the ligand absorption and the homogeneity of the mixtures. The other two wavelengths are used to print a table of the differences and to plot the titration curves. For the first wavelength the noise is printed (see Sample Result).

The 'Mode' function is used to select either a mode where the reference measurement is performed with air or a mode where the reference is measured every time before titration (see HP 8451 Owner's Manual). The 'Volume' function is used to enter the total volume in the cuvette (in μl) and the total volume of the titrator syringe (in μl).

The subroutines of the BITRATE software exchange the measurement parameters as well as flags, counters, and spectra by means of a COMMON data block (Appendix C).

7.4.3.2 Measure Subroutine

The major function of the measure subroutine is the acquisition of an array of spectra. Each spectrum is measured after the addition of an aliquot of ligand to the enzyme. For this purpose the subroutine has to be able to control both the photometer and the stepper motor. Prior to measurements the syringe has to be filled and equilibrated from the ligand stock solution. Especially important is the removal of all air bubbles that might have been caught in the syringe. This is done by moving the syringe piston backwards and forwards with the functions fill and empty of the syringe control menu.

The Agla syringe is moved in the syringe control menu, a third level menu. It can be accessed from the bitrate menu, a second level menu, with the softkey 'Syringe'.

10.1	Fill	Empty	500.0
Return	Out	Suck	Amount

This menu solely changes the two lines at the bottom of the screen. The 'OUT' and 'SUCK' functions are used to move the amount displayed in the field at the left of the upper line (μl) in and out each time they are pressed. This amount can be changed with the 'Amount' Key in increments of 0.1 μl . The function 'Return' is used to return to the calling menu or subroutine. The number in the right field of the upper line displays the fill level of the syringe in μl .

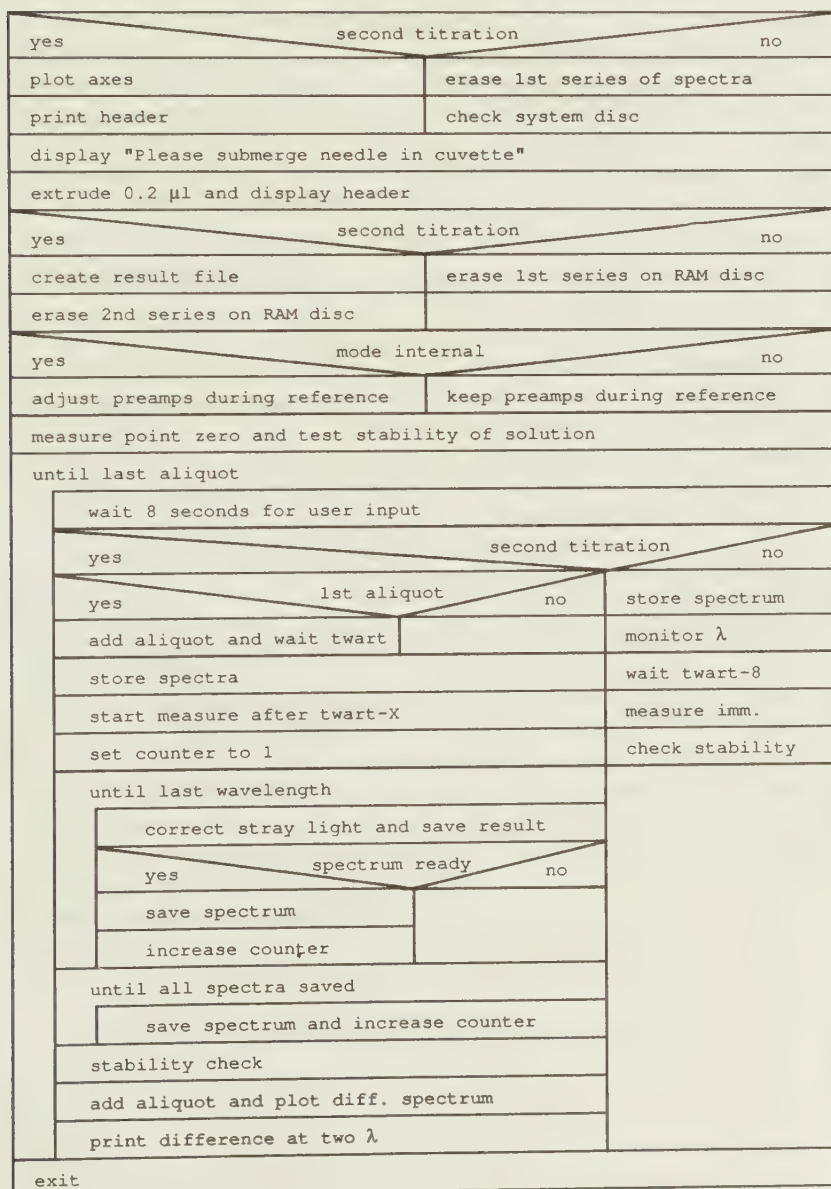


Figure 34: Nassi Shneiderman diagram of BITRATE data acquisition

Another possibility to move the syringe is the 'Wash' function in the bitrate menu. Here the number of cycles have to be entered (0.5 - 20). The piston is moved back and forth in order to rinse the syringe.

A binding titration experiment is executed by the function 'Bitrate' (softkey # 4 in the bitrate menu). Before the titration can be started, a filename has to be entered (4 characters). This name is extended by a character (A-Z) and a number (0-9). The user is guided by messages displayed on the screen. After having performed the desired operation the next step is started by pressing the ('cont') key.

The acquisition of a titration curve is done with the following steps:

- 1.* The light intensity (I_0) of the lamp is measured without anything in the light path. Here, the stability of the lamp is checked.
Message : Please keep cuvette holder free (+cont)
- 2.* The stray light (I_s) of the photometer is measured with a black log in the light path. If the stray light is too large, the lenses have to be cleaned or the lamp has to be replaced.
Message : Please put a black log into the light path.
Error : Light intensity is insufficient.
3. A cuvette with an exactly weighed amount of buffer and equipped with a magnetic stirrer is put into the light path. The spectrum is measured and stored (A_b).
Message 1 : Please keep light path free (+cont)
Measuring baseline
Message 2 : Please put cuvette with buffer into the light path (+cont)
Measuring buffer absorption
4. The Agla syringe is filled out of a perfectly homogeneous and dust free stock of ligand. The syringe tip is cleaned and submerged into the cuvette.
Message : Please submerge syringe tip in cuvette (+cont)
Start of first titration
5. A first series of n aliquots is added, the spectra are measured and stored (A_{1j}).
6. A cuvette with exactly the same amount (step 3) of enzyme solution equipped with a magnetic stirrer is put into the light path. The spectrum of the enzyme is measured, corrected and stored (A_{cE} , see next Section). During this step the stability and purity of the enzyme solution is checked.
Message 1 : Please put cuvette with buffer into light path (+cont)
Measuring baseline
Message 2 : Please put cuvette with enzyme into light path (+cont)
Measuring enzyme
Message 3: Please move syringe tip to ligand stock solution (+cont)
Filling syringe
Message 4 : Please submerge syringe tip into cuvette (+cont)
Start of second titration
7. Repeat step 4

8. The identical series of n aliquots is added, the spectra are measured, corrected (Ac_{1i}, Ac_{2i}), the difference is calculated (Δ_i) and stored.

* Steps 1 and 2 have to be performed only once a day after the photometer has been allowed to warm up for 1-2 hours. The other reason for repeating these steps is a change of the main wavelength range.

7.4.3.3 Correction of Systematical Errors

A photometer measures the amount of light (I) that passes the cuvette and compares it to the light emitted by the lamp (I_0).

$$\text{Abs} = \log\left(\frac{I_0}{I}\right) = \epsilon * c * l \quad (1)$$

The light is measured with a photomultiplier or a photodiode. These electronic devices convert light intensity to current or voltage. But they are subject to a certain noise level; the output voltage is not zero even if no light reaches the detectors, this is called the **stray light** (I_s). This noise is very small; under most conditions it can be neglected. But if small differences have to be detected at different light intensities (I), a correction is essential. Taking into account the stray light, equation 1 has to be rewritten in the following form :

$$\text{Abs} = \log\left(\frac{I_0 + I_s}{I + I_s}\right) \quad (2)$$

Equation 1, the Lambert Beer's law, is only true as a first approximation. Thorough analysis of spectroscopic titrations revealed a second term with quadratic dependence on ligand concentration :

$$\text{Abs} = \epsilon * c * l + \epsilon_2 * c^2 * l \quad (3)$$

Thus, the correction of stray light alone could not eliminate all sources of systematical errors. But all errors are small at low concentrations and increase steeply at high concentrations. After correction of stray light, the difference between the theoretical absorption and the measured absorption was reduced by a factor of two. The residual difference showed the same ligand dependence as the uncorrected difference (see Figure 36 B).

In theory, the absorption has to be converted to light intensity (I) and the stray light has to be subtracted from both I and I_0

$$A_c = \log\left(\frac{(I_0 - I_s) * 10^{(A + A_b)}}{I_0 - I_s * 10^{-(A + A_b)}}\right) - A_b \quad (4)$$

As I_s is very small compared to I_0 , equation 4 can be simplified to :

$$A_c = \log\left(\frac{10^{(A + A_b)}}{1 - 10^{-(A + A_b)} * (I_s / I_0)}\right) - A_b \quad (5)$$

The ratio I_S/I_O is measured with a black log in the light path. By using mock up titrations, a correction factor for the ratio I_S/I_O is determined (see Test Section) that allows full correction of systematical errors with one equation : $F_C = I_S/(I_O * \text{factor})$

$$A_C = \log \left(\frac{I}{1 - I * F_C} \right) - A_B \quad (6)$$

All spectra are corrected this way. The corrected absorption difference is then calculated, plotted, and stored in an array on disc.

$$\Delta_i = [A_{C2i} - A_{CE} * \frac{V_O}{V_O + \Sigma a_i} - A_{C1i}] * \frac{V_O + \Sigma a_i}{V_O} \quad (7)$$

7.4.3.4 Data Transfer Subroutine

The main function of this subprogram is the transmission of data via the RS-232 interface to a host computer. Data files that have been stored on disc are read in and transferred to the host. When the softkey 'Master' is selected in the data transfer menu, the data transfer is executed by this subroutine. In this case the data transfer program of the host has to open a file when the data transfer routine of the photometer sends an 'OPEN' command and a filename. The file has to be closed when a command word 'CLOSE' is sent.

The data transfer has to be performed by the host computer if the softkey 'Slave' is selected. In this mode the command 'E' exits to the dacom menu. The command 'D' sends the directory of the disc to the host. The command 'T' activates a routine which inputs a filename and sends the file to the host. The structure of the transferred data is listed in Appendix C.

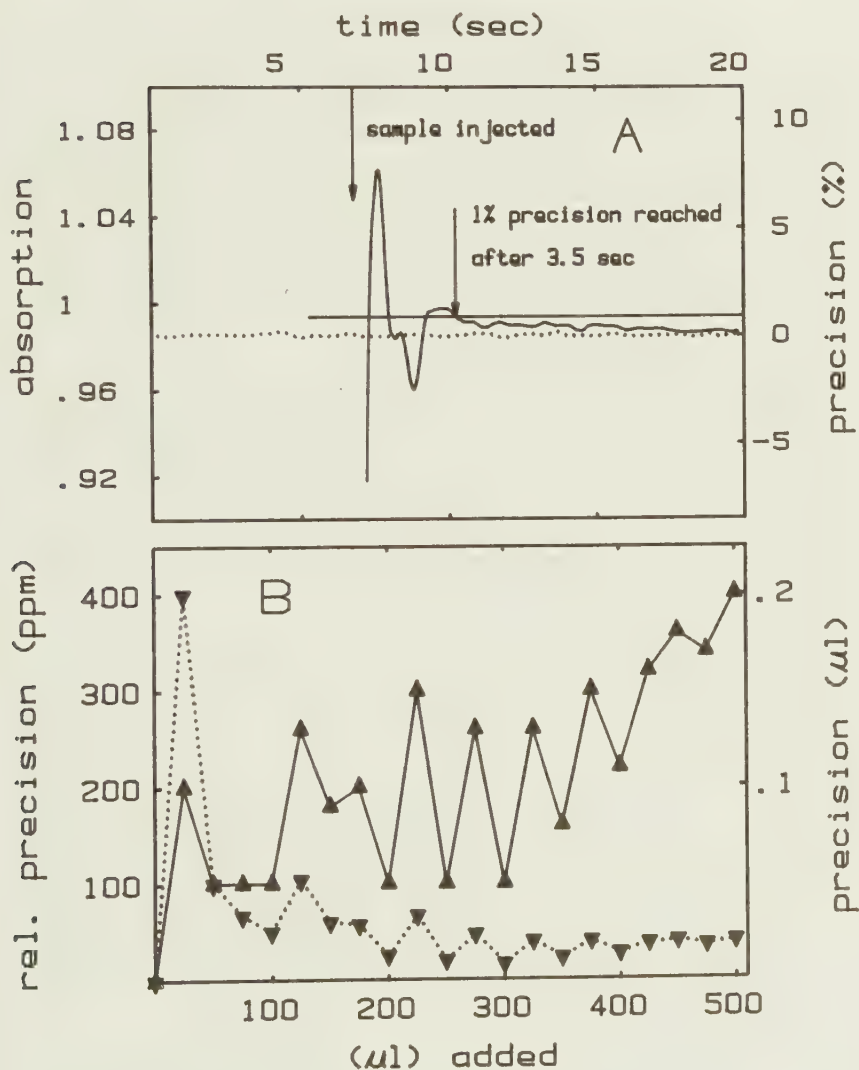


Figure 35: Efficiency of magnetic stirrer and precision of Agla syringe

Panel A : Optical density after injection of sample (—, 0-20 sec), (·····, 20-40 sec). Mixer driven by cooling water.

Panel B : (▲, —, right ordinate) absolute precision (in μl) of Agla titration. (▼, ·····, left ordinate) relative precision of Agla titration in ppm.

7.5 Test of Binding Titrator

Before the binding titrator could be used to determine dissociation constants, it had to be tested thoroughly. Especially the routine correcting the systematical errors had to be tested by mock up titrations. The results obtained using the binding titrator were compared with results measured with the four cell method, with fluorescence quenching, and with the method of Hummel and Dryer. Several problems had to be solved during the test phase: Inaccurate initial volume, bleaching of enzyme solutions by ultraviolet light, errors due to dust particles and gas bubbles, and shifts due to lamp fluctuations. The following paragraphs summarize the performance of the binding titrator in the configuration that was used for the acquisition of all spectroscopic data.

a) Stability of the photometer

The stability of the single beam HP 8451 photometer was tested with filters and cytochrome c solutions for 1 hour periods. Even after more than 4 hours of warm up the spectra shifted more than 0.005 OD during 1 hour (Figure 35 A, solid line). This was insufficient for binding titrations.

The shifts could be corrected by using an internal reference wavelength range. The spectrum was set to zero at this range. The stability achieved by this correction was better than ≈ 0.001 OD per hour (Figure 35 A, dotted line).

b) Accuracy of mixtures

The HP 8451 was equipped with a cell stirring module (Part No. 89054A) below the thermostatable cell holder. This provided magnetic induction stirring inside the cuvette. The efficiency of the stirring was tested by addition of cytochrome c (1 OD at 410 nm). The time course of the optical density at 410 nm was recorded vs. time. Connection of the water driven stirrer directly to the water bath resulted in inefficient mixing; 1% stability was reached after 15". Driving the stirrer separately by cooling water was much more efficient. Figure 35 A shows that the 1% stability was reached in less than 5 seconds.

The precision of the Agla syringe was tested by pipetting several series of aliquots into a cuvette on a Mettler analytical balance. The systematic deviation from 500 μ l was less than 0.2 μ l. More important was the check of reproducibility under identical pipetting conditions. The reproducibility of volume delivery as a function of added volume is visualized in Figure 35 B.

c) Correction of systematical errors

A titration of a ligand into a colorless buffer is different from an identical titration into a buffer with an inert colored substance. Figure 36 B (dotted line) shows the difference between two titrations of 0 - 10 μ M cytochrome c into a buffer once with and once without potassium ferricyanide (1 AU at 410 nm). The efficiency of the stray light correction was tested with the same mock up titrations of cytochrome c into buffer and ferricyanide solutions of different optical densities. In order to exclude any effect of ferricyanide on cytochrome c, some ferricyanide was added to the cytochrome c stock. The absolute error

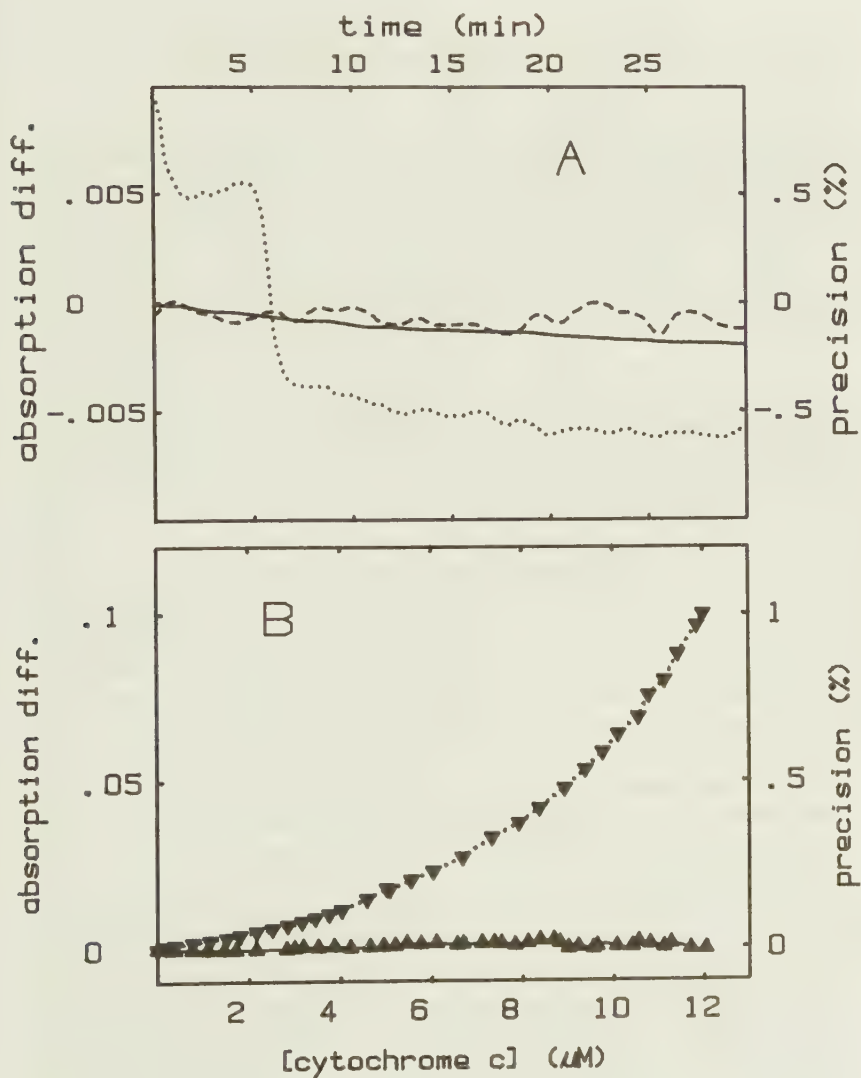


Figure 36: Stability of photometer and correction of systematical errors

Panel A : (—) Stability of HP 8451 photometer without sample, (·····) stability of mixed solution at 1 AU, (-----) stability of mixed solution with internal reference at 1 AU.

Panel B : (∇ , ·····) uncorrected absorption difference during mock up titration of cytochrome c into ferricyanide (1 AU at 410 nm), ($\blacktriangle$, —) absorption difference corrected with stray light correction routine (see Text).

never exceeds 0.0015 absorption units if the total absorption is kept below 2 OD (Figure 36 B, solid line).

d) Stability of enzyme solutions

The optical properties of fully oxidized ferricytochrome c are very stable at neutral pH. It is more stable than inorganic dyes in solution. For this reason cytochrome c was used to check the effect of prolonged irradiation on enzymes. Exposure to the full wavelength range of the deuterium lamp caused severe bleaching of the solution. For that reason the filter wheel in the position II or III (see HP 8451 Owner's Manual) was used to remove wavelengths lower than 250 nm. Without the far UV, cytochrome c remained stable at ± 0.005 OD during 1 hour when an internal reference was used.

When the cuvette was left open, however, the absorption increased slightly (approximately 0.5%) during one hour. The reason for this increase of absorption was a loss of solution due to evaporation in the cuvette. This evaporation could be measured on an analytical balance using a cuvette with a surface of 1 cm^2 ($13.4 \text{ mg}/(\text{h cm}^2)$ at 20°C). Systematical errors due to evaporation of water were neglected in the calculation of dilutions. But care was taken that the duration of the first and the second titration was identical.

Dust particles and small gas bubbles greatly decreased the accuracy and stability of the measurement. In most cases gas bubbles could be prevented by degassing buffer, enzyme solution, and the ligand solution shortly before the measurement. Dust particles could be removed by filtering the solutions with $0.2 \mu\text{m}$ disposable filters. The cuvettes were washed before the measurement with distilled water and dried with a stream of nitrogen (not lens cloths!). The magnetic stirring rods too had to be washed and dried with nitrogen shortly before the measurements. Recontamination of the dust free solutions and cuvettes was prevented by a cover.

Most stability problems could be minimized when the measurement was made faster by automatization and when not more than 40 titration points were performed.

e) Reproducibility of titrations

The reproducibility of titrations was tested with a cytochrome c solution ($50 \mu\text{M}$) that had been fully oxidized by addition of 0.1% (mole/mole) of oxidase.

The Agla syringe was filled with $500 \mu\text{l}$ of $50 \mu\text{M}$ cytochrome c and a cuvette with $2000 \pm 0.2 \mu\text{l}$ of 10 mM phosphate buffer pH 7.4 was fixed in the thermostated cell holder. Cytochrome c was added in aliquots of $50 \mu\text{l}$. 15 seconds after each addition a spectrum was measured and stored. The cuvette was refilled, an identical series of aliquots was added, and the optical density was compared to the first series. The reproducibility of these tests were better than ± 0.005 OD for all additions.

f) Initial volume

Most important for an accurate spectroscopic titration is the proper determination of the initial volume (V_0). Attempts to achieve satisfying accuracy with several types of pipettes failed:

- quick pipettes	2000 +/- 4 µl
- Hamilton syringes	2000 +/- 2 µl
- constriction pipettes	2000 +/- 1 µl

For that reason the initial volume had to be determined by weighing on a Mettler analytical balance. To do this, the volume had to be multiplied by the density of the buffer at 20°C. If buffer with an ionic strength of 25 mM (15 mM NaCl) was used the density was close to 1 so that no correction was necessary. Buffers with ionic strength of 50 mM (40m NaCl) and 75 mM (65mM NaCl) were corrected however, the factors were 1.0007 and 1.0033 respectively. As a rule the weight of the buffer was determined with an accuracy of 2000 +/- 0.2 mg. The absolute weight was less important than the reproduction of an identical pair of volumes.

g) Reliability of results

The reliability of the results was tested with cytochrome c peroxidase. This enzyme has been used by Erman and Vitello (1980) for manual determination of difference spectra and dissociation constants. The dissociation constant that was determined with the binding titrator (0.1 µM) was within the range of the scatter of their results (0.16 +/- 0.08 µM). The maximal difference extinction coefficient of their method ($\Delta\epsilon_{\text{max}408} = 4.3 \pm 0.2 \text{ mM}^{-1}\text{cm}^{-1}$) was similar to the maximal difference extinction coefficient determined with the binding titrator ($\Delta\epsilon_{\text{max}408} = 3.9 \pm 0.6 \text{ mM}^{-1}\text{cm}^{-1}$).

7.6 Sample Result

In this section the steps for the acquisition of spectroscopic titrations are summarized. Especially own experience and solutions to problems are pointed out. In addition to that the exact meaning of entries in the protocol is described.

Before the photometer is switched on, the IEC bus has to be connected to disc drive and stepper motor controller. Then the disc drive is switched on, the system disc is inserted and the photometer is started (see Main Program).

In this main menu the measurement parameter file is read in (see measurement parameter menu) and the subroutine is started. Before the measurement can be started, the photometer has to warm up for 1 to 2 hours. This time can be used to prepare the solutions (degassing, filtering, see below) and to fill the Agla syringe (see Measure Subroutine).

Immediately before the titration can be started a cuvette has to be filled on a analytical balance with an exact amount of buffer (mostly 2.0000 ml taking into account the density of the solution).

The titration is started with the 'Bitrate' softkey in the bitrate menu. The user is then guided through the measurement by messages displayed by the softkey (see page 132).

A typical printout of a spectroscopic titration consists of several parts:

- a) The first two lines contain the filename, the date of the measurement and the volume name of the disc.
- b) All important measurement parameters are printed together with two lines of comment that can be entered just before the measurement.
- c) The next block shows the optical density together with the standard deviation of five titration points at the monitoring wavelength (see measurement parameter menu). If 40 titration points have been selected, points no. 0, 10, 20, 30, and 40 are printed; the other points are displayed on the screen during the measurement.

The monitoring wavelength should be selected at the absorption maximum of the titrated ligand because the noise at this wavelength is used to check the efficiency of stirring in the first and the second titration. If the standard deviation exceeds the value in the parameters (Figure 37, fifth line), the measurement is repeated. The difference spectra are best if the optical density of the ligand is kept below 1 AU.

- d) In the next block the absorption differences at two wavelengths and the noise at the monitoring wavelength are listed. In this example the difference is calculated at 410 and 416 nm. The titration curves can be selected at any wavelength within the titration wavelength range (390 - 450 nm in Figure 37). The first line of the list (No. 0) has been measured with a 15 second delay after the baseline. It is useful to verify that the absorption of the enzyme is stable (only large instabilities are rejected by the test routine). The following lines list the differences after each addition of 10 μ l of ligand to the enzyme.
- e) On the second page the storage location and the two lines of comment are repeated. After this the difference spectra of the titration range (here 390 - 450 nm) are plotted. Then a second plot shows the absorption difference at two wavelength (410, 416 nm) as a function of ligand concentration. (The ligand concentration is calculated from the concentration in the stock solution).

Spectroscopic titrations with absorption differences less than 0.02 AU at a total absorption of 2 AU should be repeated three times in order to make the result more reliable. Direct curve fits to averages of several titrations are made easy by the MICHFIT software as averages are performed during input of curves. The accuracy of spectroscopic titrations with optically slightly instable ligands can be increased if the second titration is performed twice, once with enzyme and once again into buffer. If the second titration into buffer is not zero, it can be used to correct the titration curve with the enzyme.

Titration :	CHIK A 3	A
21 Okt. 86	Volume : Amanda	

Duration of measurement	12 sec	B
Waiting before	15 sec	
Maximal noise	0.005 AU	
λ -range titration	390 to 450 nm	
λ -range spectra	390 to 630 nm	
λ -range int. refer	620 to 630 nm	
Oxidase VIA Conc.	2.8 μ M	
Chicken Cyt c Conc.	45 μ M	
Number of points	40	
Initial volume	2000 μ l	
Stray light factor	18	
Data format 1	Mode : 1	
Number of stored full spectra	6	
Test measurement with Chicken cytochrome c in 0.2% Tween-80..		

No.	Abs. 410	Noise	C
0	-0.00008	0.00012	
10	+0.22768	0.00024	
20	+0.42899	0.00042	
30	+0.61128	0.00056	
40	+0.93066	0.00130	

No.	Titrr.410	Noise	Titrr.416	D
0	+0.0002	.00041	+0.0003	
1	+0.0013	.00053	+0.0003	
2	+0.0024	.00056	+0.0010	
3	+0.0038	.00090	+0.0019	
4	+0.0056	.00076	+0.0036	
5	+0.0072	.00059	+0.0050	
6	+0.0088	.00068	+0.0061	
7	+0.0104	.00072	+0.0080	
8	+0.0119	.00063	+0.0096	
9	+0.0130	.00083	+0.0106	
10	+0.0143	.00087	+0.0116	
11	+0.0152	.00071	+0.0124	
12	+0.0160	.00096	+0.0126	
13	+0.0167	.00065	+0.0137	
14	+0.0166	.00100	+0.0134	
15	+0.0173	.00080	+0.0139	
16	+0.0169	.00085	+0.0138	
36	+0.0201	.00210	+0.0154	
37	+0.0203	.00310	+0.0141	
38	+0.0217	.00240	+0.0147	
39	+0.0209	.00270	+0.0147	
40	+0.0212	.00180	+0.0162	

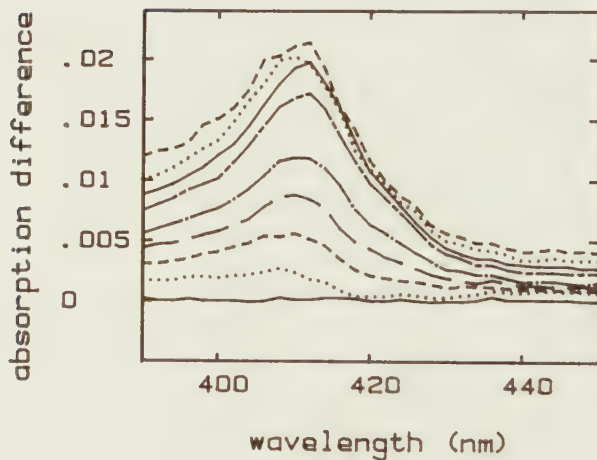
Figure 37 : Sample spectroscopic titration (1)

Titration : CHIK A 3
21 Okt. 86 Volume : Amanda

E

Test measurement with Chicken
cytochrome c in 0.2% Tween-80..

F



G

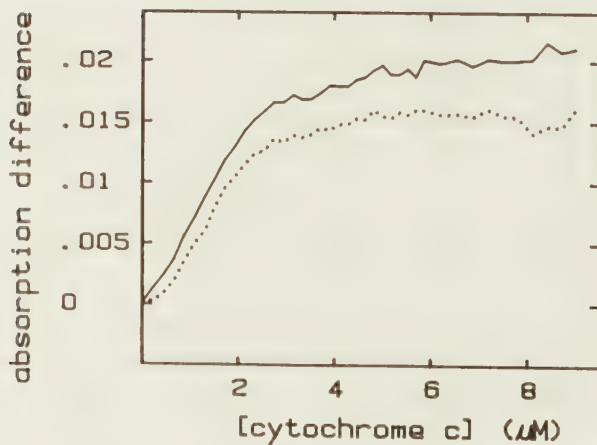


Figure 37 : Sample spectroscopic titration (2)

Figure 37: Sample spectroscopic titration

- A: Header with filename, date of measurement and name of volume.
- B: Measurement parameters with 2 lines comment (the function of the measurement parameters is explained in the Main Program Section).
- C: Absorption (and noise) of monitor wavelength during first series of titrations. Only five points are printed, the other points appear on the screen during the titration.
- D: Absorption difference at two wavelengths calculated during second series (410 and 416 nm). Not all 40 data points are shown here. Middle column: noise at first wavelength (410 nm). The noise at the second wavelength together with the absolute absorption is displayed during the second titration.
- E: Header with filename, date of measurement and name of volume together with 2 lines of comment (for second page).
- F: Difference spectra after the addition of (——) 0, (·····) 20, (-----) 40, (— — —) 60, (— · — · —) 80, (————) 160, (·····) 240, (-----) 340, and (— — —) 500 μ l of ligand (here cytochrome c).
- G: Plot of titration curves (Absorption difference vs. ligand concentration). (——) absorption difference at 410 nm, (·····) absorption difference at 416 nm.

chapter 8

THE ASSAYOMATE

8.1 Introduction

In clinical laboratories photometric steady-state assays * are used to measure a large number of enzymes and substrates in body fluids and tissue samples. Many sophisticated automatical devices have been developed to measure thousands of probes per day. The fastest devices use the continuous flow technique. They can perform up to 36'000 assays per hour. But the setup for such a complicated apparatus lasts several days and the measuring range is very narrow. Other devices use principles that mimic the manual assay mixing mode with robots. These devices too have a large throughput but they can be used only for a limited number of standardized assays. All these devices are complicated to setup and they measure enzyme reactions at a single substrate concentration only.

Steady-state assays are widely used in biochemical research. Up to the present day all assays are mixed by hand. This includes pipetting of buffer and substrate into the cuvette and starting the assay by the addition of 5 or 10 μl of enzyme and manual mixing of the content of the cuvette. Then the photometer is activated and the optical density is monitored for 20 up to 60 seconds. The time that elapses between mixing and starting the measurement, the dead time, is at least 5 seconds. The velocity of the enzyme reaction is obtained by drawing a tangent to the absorption versus time curve and the steady-state turnover is calculated from the slope of the tangent.

Very fast reactions cannot be measured by this method; therefore **stopped flow** devices were developed, where assays are mixed mechanically by use of two equal syringes, one with substrate, the other with enzyme (Figure 38). The pistons of the syringes are pushed forward simultaneously by compressed air, the solutions are mixed by turbulence in a passive mixer and transported into a cuvette. The flow is stopped and the measurement is started by a stop syringe. With stopped flow dead times of less than 5 milliseconds can be achieved. But there are still a lot of manual operations necessary: The stop syringe has to be emptied by hand and the time course of the optical density has been analysed conventionally from a photograph of the oscilloscope after each measurement. The syringes have to be rinsed and refilled by hand for each change of substrate or enzyme concentration.

The general principles of chemical reaction kinetics apply to enzyme-catalyzed reactions, but they also show a distinctive feature not usually observed in nonenzymatic reactions **saturation** with substrate. At low substrate concentration the initial reaction velocity (v) is nearly proportional to the substrate concentration. However, as the substrate con-

* An assay is a mixture of buffer, substrate and enzyme produced for the purpose of measuring certain properties of an enzyme, or concentration of metabolites.

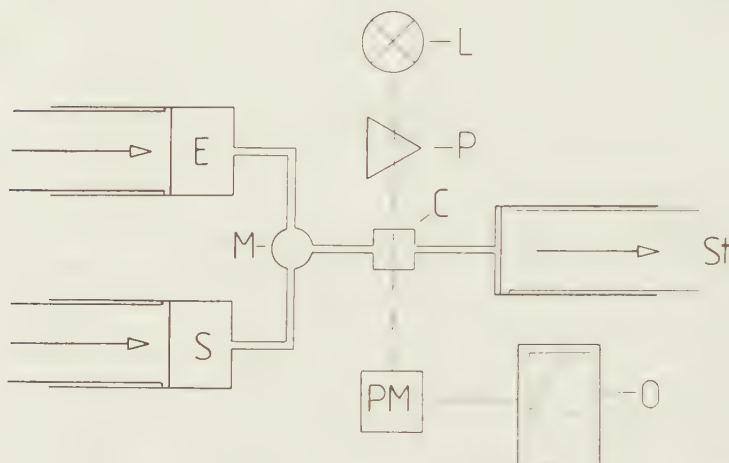


Figure 38: Scheme of stopped flow apparatus

E=enzyme, S=substrate, L=light source, P=prism, C=cuvette, PM=photomultiplier, O=oscilloscope, St=stop syringe

centration is increased, the reaction becomes independent of substrate concentration and asymptotically approaches a constant rate ($V_{\max}$). The Michaelis - Menten theory assumes that the enzyme E first combines with the substrate S to form the enzyme-substrate complex ES; the latter then breaks down in a second step to form free enzyme and product P.



The Michaelis - Menten equation (Michaelis and Menten, 1913) is the rate equation for reactions catalyzed by enzymes having a single substrate.

$$v = \frac{V_{\max} * [S]}{K_m + [S]} \quad (9)$$

v is the reaction velocity (change of substrate or product concentration per unit time) measured at concentration $[S]$ of free substrate. Usually $[S]$ is much larger than the enzyme concentration and can be set equal to the total substrate concentration. $V_{\max}$ is the maximal velocity the enzyme can achieve. K_m is the Michaelis constant. Equation (9), the Michaelis Menten equation, is a fundamental equation in enzyme kinetics; it is derived and discussed in every textbook of biochemistry. It is obvious that v never reaches $V_{\max}$ at finite substrate concentrations. It is thus not correct to estimate $V_{\max}$ by measuring the velocity v at one substrate concentration only! K_m is the substrate concentration where the enzyme reaches 50% of its maximal velocity. K_m and $V_{\max}$ and other kinetic constants can only be determined correctly from a series of measurements at different substrate concentrations (see Introduction on page 115).

Up to now all kinetic constants had to be determined by hand. Because kinetic constants are determined in many laboratories, such a process is an ideal candidate for automation :

- It is often necessary to measure the kinetic constants of an enzyme as a function of pH, ionic strength, inhibitors, activators or a second substrate.
- The proper determination of such a dependence needs hundreds of assays, this is very time consuming and tedious.
- Mixing different substrate concentrations is subject to errors.
- As enzymes are often unstable, errors result if the measurements last too long.
- Repetitive measurements yield useful statistic information on the accuracy of the result.
- Screening of a large number of samples becomes easy.

8.1.1 Aim

The aim of the work presented here was the design and construction of an automatical assay measuring process.

With this process it should be possible to determine the kinetic parameters K_m and V_{max} fully automatically within less than 15 minutes.

This goal was to be achieved by the construction of an assay mixing robot, the **Assayomate**, with the following characteristics :

- Three syringes driven by independent stepper motors should allow complicated mixing sequences without changing the stock solutions.
- Carefully thermostated stock solutions, to avoid temperature artefacts on a wide temperature range.
- Short dead time compared to manual operation (but the measurement of fast kinetics should not be the main goal of the design).
- It should be controlled via an IEC bus from a computer which performs the acquisition and evaluation of the spectroscopic data.

The software should ease the acquisition of kinetic parameters as much as possible:

- The Assayomate should calculate the need for substrate, enzyme and buffer and should fill the syringes automatically.
- A whole series of assays at different substrate concentrations should be mixed and measured without manual intervention.
- After the last assay the kinetic constants should be calculated, printed and stored on disc.

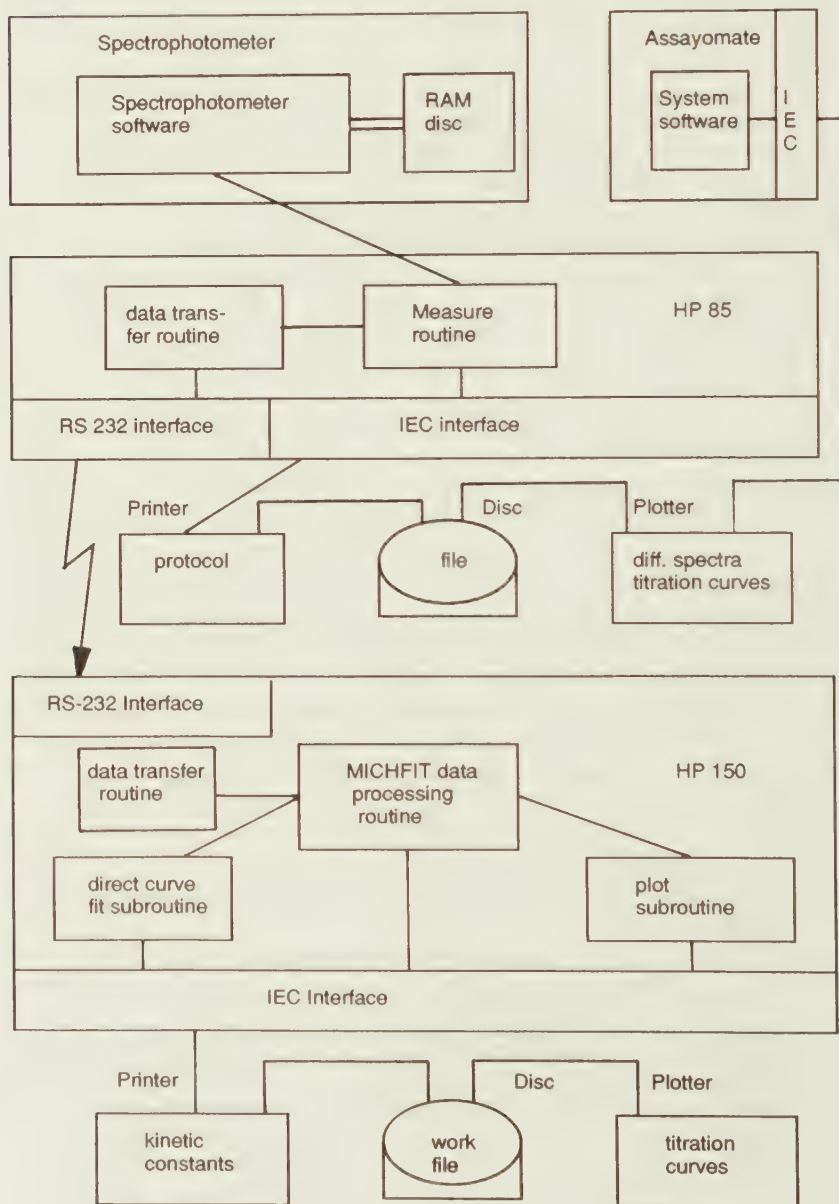


Figure 39: System concept of MICHKIN software

8.2 System Concept

The complete system for the automatic determination of kinetic constants is shown in Figure 39. It is composed of four parts : (i) the spectrophotometer, (ii) the Assayomate, (iii) the computer (HP 85) controlling the photometer and the Assayomate, and (iv) a host computer for processing and presentation of data. A printer, a plotter, a disc drive, and the Assayomate are connected to the HP 85 by means of an IEC bus (see Figure 40).

The spectrophotometer is equipped with a Z-80 central processing unit. The spectrophotometer system software is responsible for the acquisition of spectral data, for temporary storage of these data in the RAM disc and for the transmission of the data to the HP 85 computer.

The Assayomate is equipped with a Z-80 central processing unit too. The Assayomate system software is responsible for mixing assays and for the management of buffer, enzyme, and substrate stocks.

The MICHKIN software controls the photometer and the Assayomate. It is written in HP BASIC and runs on a HP 85 computer with additional system software (see Software Section). It calculates derivatives of absorption versus time data, averages the results of several assays and calculates Michaelis constants and maximal turnover numbers from the experimental data with a linear regression routine.

For a direct curve fit and for graphical presentation, the data has to be transferred to a host computer (HP 150) by means of an RS-232 interface. The MICHFIT software fits a wide range of kinetic models to the experimental data. Simulated curves generated from the results of the direct curve fit routine are graphically presented together with the experimental data with the plot subroutine. Data and simulated curves are stored in workfiles or in plot files (see MICHFIT Section).

The operation of the system is controlled by the measure subprogram of the MICHKIN software. A series of assays can be initiated by the user by pressing the 'Km Meas' softkey in the 'Measure menu'. The command is converted to the format needed by the Assayomate, sent by means of an IEC bus, and the status of the Assayomate is checked. The operating system of the Assayomate interprets the command, and mixes the first assay. A fraction of a second before the assay has been finished, the Assayomate clears the busy flag. This is detected by the measure subprogram and a measure command is sent to the photometer controller. The photometer opens the shutter and samples the light intensity of 10 photodiodes in increments of 100 milliseconds, and sends the result to the computer. The change of optical density per second is calculated by the measure subprogram and compared to a reference value. The amount of enzyme is adjusted to reach the preset change of optical density per unit time, a series of assays is measured at the newly fixed enzyme concentration, and the results are printed and plotted. After the termination of the series of assays, the results are converted to turnover numbers, printed, plotted, and stored on disc. The result may be sent to the host computer at a later time by the data transfer subroutine.

8.3 Structure of the Hardware

Substrates of enzyme reactions are mostly quite expensive. It is therefore desirable to miniaturize the assay as much as possible without losing accuracy. The most important factors determining the size of an assay are the cuvette, the mixing chamber and the connection between them. It is most important to very carefully design these components. The accuracy of the assays is reduced by too long tubes because the tubes as they are slightly flexible and offer resistance to the flow. The overall length of the tubing was kept to a minimum. Generally, much effort was spent to design the whole system as compact as possible. As construction and maintenance of modular systems is much easier, the Assayomate was designed in 9 totally separate modules. Each of those modules can be easily unscrewed and unplugged.

- 1 - Electronic module (EM)
- 2 - Power supply module (PS)
- 3 - Keyboard - display module (KD)
- 4 - 3 syringe modules (SM1) (SM2) (SM3)
- 7 - Valve module (VM)
- 8 - Mix - measure head (MM)
- 9 - Autosampler (AS)

It is not within the scope of this thesis to show mechanical construction drawings or electronic circuits. The user of the MICHKIN software and the Assayomate is referred to the 'MICHKIN User manual' and 'Assayomate User Manual'.

8.3.1 Electronic Module

The Z-80 processor was selected as the central processing unit (CPU) for three reasons : (i) it uses a powerful instruction set (158 instructions), (ii) it is used in many similar applications, and (iii) complete Z-80 systems need only a small number of electronic components. Moreover, new Z-80 versions are now available operating at higher speed (4 and 6 MHz), and Z-80 development systems are not too expensive.

The Z-80 is an 8-bit processor with an 8-bit data bus and a 16 bit address bus. It is capable to address 64 kBytes (65536 of $FFFF_{16}$ Bytes) of memory. The lower addresses (0-FF, 8 bits) are used in two modes, in the memory mode (MREQ=true) or in the input-output mode (IOREQ=true) to address interfaces to peripherals. This technique is called IO-mapping.

During power on, or after a reset, the Z-80 sets the program counter to zero and starts executing the commands while incrementing the program counter. For this reason the lowest addresses in memory are occupied by the start up routines, by interrupt routines and the operating system, fixed in read only memory. The highest addresses ($F800-FFFF_{16}$) are used for the system variables, for input output buffers, for the screen and for the system stack; these addresses have to be occupied by RAM. During the start up the default screen and the default system variables are copied from ROM to RAM.

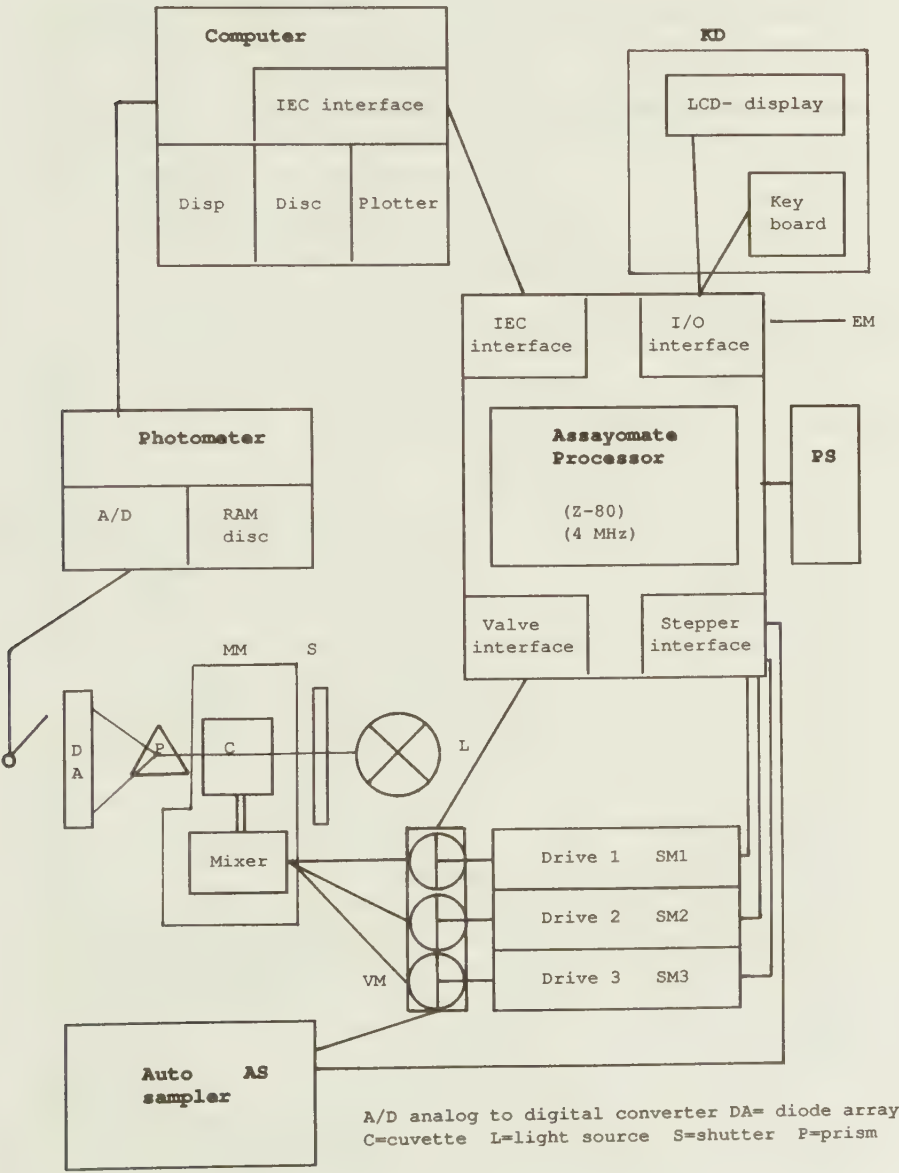


Figure 40: Survey of Assayomate hardware

8.3.2 Display - Keyboard Module

The keyboard is built up as a matrix of 8 columns and 4 rows. Key presses are detected by sequentially setting one row to zero and analyzing the logical state of the columns. The position code of the key is converted into ASCII codes with a conversion table. If more than one key is pressed at a time, the input is ignored.

The display is a complete module. It displays 2 lines of 40 characters composed of a 5 x 7 dot matrix.

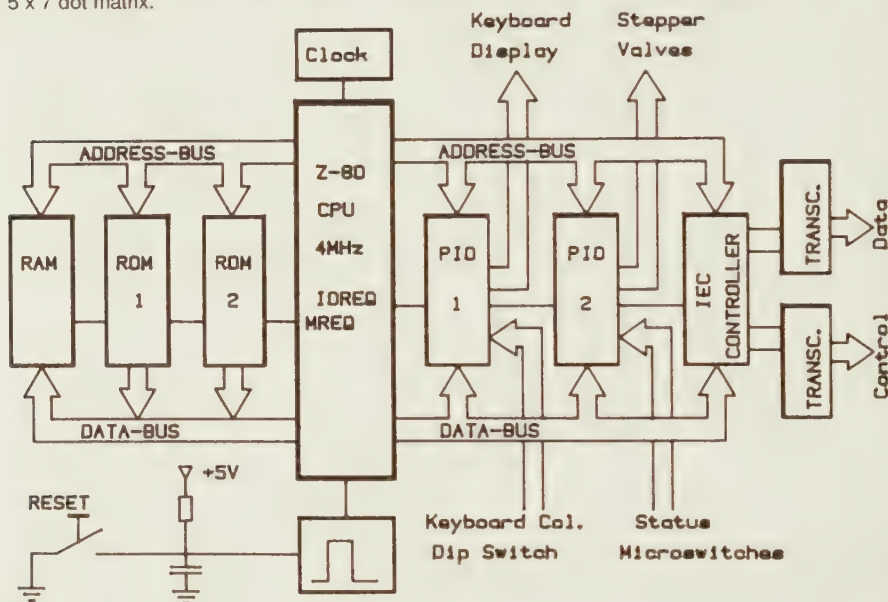


Figure 41: Scheme of Assayomate electronics

8.3.3 Syringe Drives and Valve Module

The solutions (buffer, enzyme, substrate) are sucked into Hamilton syringes with volumes of 50 ml, 5ml, and 2.5 ml, respectively. The temperature is controlled by a water jacket around the syringe. The jacket is connected via tubes to the rear panel of the Assayomate and from there to an external water bath. The forward movement of the piston is stopped by a microswitch at the frontmost position. This microswitch is used in a self test routine to test the proper operation of the syringe drives.

The valve module holds three valves switching the direction of buffer, enzyme and substrate flow. Prior to the construction of the valve module an optimal set of tubes with a small fluid resistance but not too large a diameter had to be selected. The fluid resistance was calculated the 'Lohms law' proposed by the Lee company, Westbrook, CT.

'Lohms' define the resistance to the liquid flow just as ohms define electrical resistance. In contrast to the electrical problems serial liquid resistances have to be added according to the equation:

$$L_T = \sqrt{L_1^2 + L_2^2 + \dots + L_n^2}$$

(10)

The total resistance in the Assayomate consists of a tube with a diameter of 0.3 mm and a length of 35 cm, (7500 Lohms), the valve with a resistance of 3500 Lohms, and another 50 cm of tube with a diameter of 0.75 mm (2400 Lohms).

Resistance to flow in tubings :

Ø	Entrance Lohms	Lohms/cm
0.3	4000	100
0.75	1400	20
1.5	4000	5

$$L_T = \sqrt{7500^2 + 3500^2 + 2400^2} = 8617 \text{ Lohms}$$

The maximal differential pressure for this resistor is 1,8 bar for a flow of 1 ml/sec.

8.3.4 Autosampler Module

The sampler uses a luer type needle to suck solutions from different types of tubes. The needle is moved by a stepper motor via a toothed belt. A microswitch is actuated when the needle has reached its highest position. The syringe tip is washed by a special chamber. This connection is used for dilutions of enzyme stock.

The Assayomate performs kinetic measurements fully automatically on several probes that are inserted in the probe tray. After the tray has been inserted in the drive, it moves with the first tube under the sampler needle, and the needle moves down. A successful move is signalled to the controlling computer by the 'next probe ready' bit in the status byte. The probe can then be sucked into the syringe with a 'SUCK' or 'WASH' command.

Three microswitches are used to monitor the operation: Microswitch 1 tests if the tray is present, microswitch 2 tests if the door is open, microswitch 3 tests if the tray has reached the last position.

8.4 Structure of the Software

The MICHKIN software is consisted of two parts:

a) Software for the Z-80

The operation of the Assayomate is controlled by a ROM based 16 K byte system software written in Z-80 assembly language. It uses 2 K byte RAM for the system stack, buffers, system variables, the storage of a 40 x 20 character screen and external programs. The system software enables the user to enter a comprehensive set of single key stroke commands, controlling all the functions of the Assayomate (see Appendix D).

b) Software for the HP 85

The MICHKIN user software was written in HP BASIC. It uses the standardized BASIC functions as well as a large number of extended functions (instead of assembly language calls) for the control of the photometer. It provides the user interface for both the remote controlled Assayomate and photometer.

8.4.1 The System Software

An important feature of the Assayomate system software is that it can accept commands from the built in keyboard and from an external computer. This makes the device very flexible. First, it cooperates with photometers that cannot be remote controlled. Second, automatic operation can be suspended at any time in order to solve problems (strong enzyme-concentrations, air bubbles in cuvette). Any kind of hook-ups during data-transfer are prevented by the robust data-transfer protocols.

After the power has been switched on, or after the reset button has been pressed, all functions of the Assayomate are tested. A first loop delays the start until the voltage of the power supply has become stable, then the input-output ports are configured and the stack pointer is initialized. Next the display is initialized and shows the first message. Then the IEC interface controller is started and the device listener / talker address is loaded. At this point the 'PWUP' byte is tested in order to detect if a 'cold' or a 'warm' start has to be performed. If only a 'warm' start is needed, the initialization procedure is stopped and the main menu is displayed. A cold start is performed when the 'PWUP' byte is set to another value than A516. The next tasks of a cold start are the test of the ROM checksum and the test of read and write access to all RAM cells. Then, all system variables are initialized to their default values and the menu mask is copied from ROM to RAM. Afterwards, the syringe pistons are moved forward until they actuate the microswitch and then back again until the switch is released. The tray and the auto-sampler are tested in a similar way. If one of the tests fails, an error message is displayed, but an attempt is made to branch to the main menu so that the error can be localized with the aid of the memory dump routines. After all self tests have been performed successfully, the Assayomate displays 'READY', clears the busy bit in the status byte and jumps to the main menu.

During normal operation the Assayomate polls the IEC interface and the keyboard if any command has to be executed. If a key press is detected, the Assayomate executes the specific subroutine written for this function. If the function needs additional parameters, a message is displayed and an input of one line of ASCII characters is expected. The input is terminated by the carriage return key (end line, 0D₁₆). The input is checked for plausibility, the numbers are converted to binary format and the function is executed. Before and after the execution of the commands, the microswitches are checked. During the execution, a message is displayed and finally the menu is updated.

An IEC command consists of a function code and an optional list of parameters terminated by a carriage return and a line feed byte.

*K 0.300,0.050,0.050 (cr)(lf)

If an IEC transmission is stopped without a carriage return, the error message 'IEC time-out' is displayed and the command is ignored. (see Piotrowsky, 1982).

If the active syringe is changed, the control block of the inactivated syringe is copied to the storage location and the new control block is copied to the active zone. The syringe control block holds 16 Bytes of information for all syringes.

ZSTA	DEFS	2	Position of piston in pulses (fill level)
MAXC	DEFS	2	Max. pulses for a full length move
WART	DEFS	2	Number of wait cycles after a pulse (1/speed)
MPK	DEFS	2	Number of pulses executed when functions B, C, or D are pressed.
MAXF	DEFS	2	Maximal fill level in pulses
INT	DEFS	2	free
VOLUM	DEFS	4	Conversion factor for pulses into volume (in ml).

Table 12: Syringe control block

Before the syringe move command is executed, the actual fill level and the new fill level are checked if they exceed limits, and the valves are switched to their proper position. After the termination of the syringe move, the fill level is calculated from the content of the piston counter, the status of the microswitches is read and the menu is updated.

The basic input output system (BIOS) of the Assayomate is written in Z-80 assembly language. It includes a set of arithmetic functions, the stepper motor routines, and a hexadecimal monitor (see Appendix D).

The menu is displayed at any time when the Assayomate is not busy by pressing the (cr or endl) key. The softkeys are displayed on the screen when the space key is pressed. The menu can be rolled up and down with the up and down arrows.

The Assayomate menu displays 8 lines of information about the state of the syringes (Figure 42, upper box). Another two lines with 8 short labels of the softkeys are displayed on the screen when the space key is pressed (Figure 42, lower box). The display window shows only two lines with 40 characters at a time. The other lines are shown by rolling the

```
***** SYRINGE CONTROL MENU *****  
PLEASE SELECT SYRINGE : KEYS 1 2 OR 3  
      SYRINGE 1  SYRINGE 2  SYRINGE 3  
TOTALVOL 50.00 ML  5.00 ML  2.50 ML  
MAXFILL  50.00 ML  5.00 ML  2.50 ML  
FILLPOS  11.23 ML  4.78 ML  0.32 ML  
AMOUNT    1.00 ML  0.50 ML  0.25 ML  
SPEED     40.00    4.00    2.00 ML/M
```

```
AMO  GIVE SUCK GIVE  WASH EMP- FILL STOP  
UNT  OUT  IN   BACK SYR. TY  SYR. FLOW
```

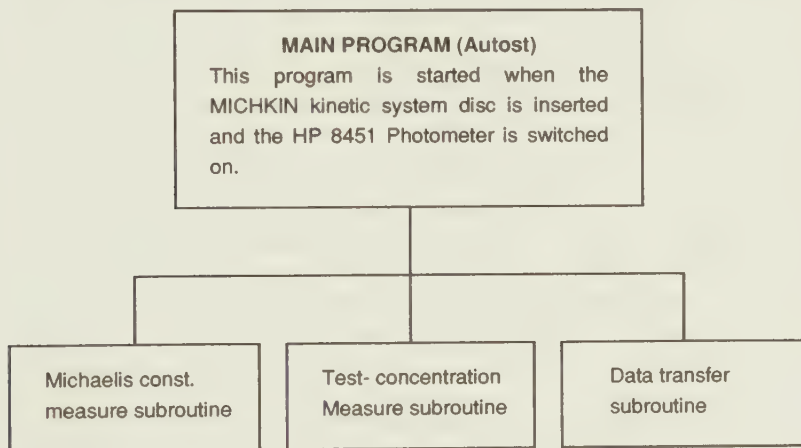
Figure 42. Menu of Assayomate

screen either up or down. The third line of the menu displays the total volume (in ml) of the three syringes that have been mounted on the drives.

The forth line shows the maximal fill level of the syringes. In response to a 'Fill' command the syringe piston moves exactly to the level shown by this field. The next line shows the volume that is in the respective syringes. Next the amount that will be moved when either a 'suck in', 'give out' or 'give back' command is executed. The line at the bottom shows the speed of the movements in ml/min.

8.4.2 The MICHKIN User Software

The user software is divided into a main program and three dependent subroutines, that can be activated from the main program. Data is exchanged between the different programs parts with the aid of a common data block. The programs have been written in HP BASIC.



Functions of subroutines

a) Main program ('Autost')

- Starts MICHKIN software package
- Initializes the COMMON data block
- Implements main menu (1st level)
- Stores measurement parameters in files
- Reads files containing measurement parameters
- Displays directories of all discs
- Purges data files and initializes data discs
- Activates subroutines
- Implements measurement parameter menu (2nd level) for input of measure parameters (e.g. λ -ranges, duration, concentrations)
- Implements syringe parameter menu (2nd level) for input of syringe parameters (e.g. fill speed, fill-level, assay volume)

b) Measure subroutine

- Implements measure menu (2nd level menu)
- Measures single assays (input of enzyme and substrate concentration)
- Changes enzyme and substrate concentrations
- Alters number of single measurements (points) and number of repetitions of points
- Measures blanks
- Measures Michaelis constants (raw data are stored on disc)
- Subtracts blanks from Michaelis constant measurements
- Measures series of enzyme probes with autosampler
- Implements syringe control menu (3rd level menu)
- Controls the syringes (fill / empty / suck in / give out / give back)

c) Test subroutine

- Implements test assay menu (2nd level)
- Tests assay conditions
- Tests series of enzymes (velocity) with autosampler
- Measures substrate and product concentrations (if they absorb UV/vis light)
- Measures spectra of substrate and product

d) Data transfer subroutine

- Implements data transfer menu (2nd level)
- Transfers files to host computer via serial interface
- Averages blank measurements
- Copies data files
- Edits data files
- Plots data (turnover number vs. substrate, double reciprocal, Eadie Hofstee or Hanes Wolfe transformation)

All subroutines are organized in a similar manner; they implement a second level menu :

measure subroutine : Michaelis menu

test subroutine : test assay menu

data transfer subroutine : data transfer menu

From the second level menus it is possible to branch to lower level menus. In all the lower level menus Softkey #1 exits from any place to the second level menu of the respective subroutine. Pressing Softkey #1 in one of the second level menus exits to the 'Main Menu', this is the highest level (first level) menu from where all operations start.

8.4.2.1 Main Program

The main program of the MICHKIN software is started automatically when the kinetic-system disc has been inserted into disc drive 0 before the HP 8451 photometer is switched on (see HP 8451 Owner's Manual). If the 'Autost' program is called for the first time all system variables and measurement parameters are loaded with a set of default values. After the completion of these tasks the initialization flag (Z8) is set, this prevents another initialization when the program is called again.

Approximately 20 seconds after the 'Autost' program has been started, a first screen is displayed. At this point the name of the data disc volume (6 characters) and the date have to be entered. The name of the volume is used to identify the data disc among several discs that are inserted in different drives connected to the HP 8451 photometer.

If the volume is found, a screen of information about the data in the files is displayed. If the disc name has been misspelled or the disc has not been initialized, an initialization menu is displayed (see MICHKIN User Manual). After the data disc has been localized, the system disc is searched for a special measurement parameter file (DEFAULT). This file, if found is read during the start up procedure. It can be edited and stored by the user like other measurement parameter files. After the initiation has been finished, the main menu is displayed. The main menu is used to read and write parameter files, to branch to subroutines, or to the measurement parameter and syringe set up menus.

Measure parameter files are read from the system disc with the function 'Read Pa' and written to the system disc with the function 'Sto Pa'. They contain all information that is necessary to determine a kinetic constant. The constants of the measurement parameter files can be changed in the measurement parameter menu. This second level menu is accessed from the main menu with the 'Meas Pa' softkey (Figure 43 A).

It is not intended to give a full description of all functions, only the functions crucial to the understanding of the software are being described. A kinetic constant can only be determined if different substrate concentration are mixed and measured. The accuracy of such a determination is best if a wide range of substrate concentrations is measured.

The function [S]max specifies the highest substrate concentration in the assay (in μM). For obvious reasons this value must not be larger than half the substrate stock concentration. But it should neither be lower than approximately a tenth of the substrate stock concentration. This can be understood when the 'Spacing' function is taken into account. The 'Spacing' defines the spacing between different substrate concentrations (e.g. arithmetical, geometrical, reciprocal, or mixed). For the geometrical spacing the lowest substrate concentration is 512 times smaller than [S]max. As it is not possible to mix ratios much lower than 1 part of substrate to 1000 parts of buffer, this limitation is obvious too. Most flexible determinations of kinetic constants is achieved with mixed spacing types (geometrical + arithmetical).

The 'Epsilon' function is used to enter the extinction coefficients of substrate and product at two wavelength ranges (defined by function ' λ -range'). Extinction coefficients

*** Measurement parameter menu 1 ***			
Enzyme name	40 nM (E)		
Substrate name	250 μ M (S)		
max. Substrate conc.	30 μ M (K)		
Range 1 from	548	to	552 nm
Range 2 from	416	to	420 nm
Int. reference	580	to	586 nm
Epsilon	Range 1	Range 2	
Substrate :	25.2	121.1	1/(mM cm)
Product :	9	78	1/(mM cm)
No of Points	20		
No. of Rep.	3	Add. Rep.	3

Conc.	Names	[S]max	λ -range
Exit	Epsilon	Points	Page 2

Figure 43: A: Measurement parameter menu (page 1)

are used to determine the stock concentration in the substrate syringe and to calculate the turnover number of the enzyme.

The 'Points' function is used to enter the number of different substrate concentrations to be measured (5 - 40) and the number of repetitions of each measurement (2 - 10). A third entry defines additional repetitions at low substrate concentrations. This function is used to compensate for the decreased accuracy of the results at the lowest substrate concentrations (see Figure 45 A).

The 'TN Scale', 'OD Sca', and ' Δ OD Sca' functions are used to define the Y-Axis of the three different types of plots that are used to display data (see Figure 43 B, Sample Result and MICHKIN User Manual).

The 'Noise' function is used to enter the maximal relative standard deviation that is tolerated when results from several assays with the same substrate concentration are averaged. If the standard deviation is too high, all assays at this substrate concentration are repeated. The second input is the maximal nonlinearity link of the time course of the optical density that is tolerated.

The 'Time W' function specifies a time window that is different for high and low substrate concentrations. The duration of the measurement has to be shorter at low substrate concentrations as the substrate is used up more quickly. Moreover it is advisable to measure all kinetic data at the same relative substrate to product ratio (see MICHKIN User Manual). The total measurement time has to be split into smaller intervals. They can be selected in increments of 0.1 sec from 0.1 to 10 sec. During each interval the data is accumulated for the time defined with the interval parameter. It should be selected between 0.1 and 0.7 sec (see MICHKIN User Manual).

*** Measurement parameter menu 2 ***											
Measure type : Michaelis in. velocity											
Time window : Mixed row											
at [S]max.	3.2	to 8	sec								
at [S]min.	1.2	to 2.4	sec								
Maximal noise 3% Max. Nonlin. 15 %											
Y-axis TN vs. [S] plot :											
0	100	20	0								
Y-axis Δ OD vs. time plot :											
-0.01	0.001	0.005	0								
Y-axis OD vs. time plot :											
-0.1	1.5	0.2	0.1								
<table><tr><td>Type</td><td>Spacing</td><td>Time W</td><td>Noise</td></tr><tr><td>TN-Sca</td><td>ΔOD-Sca</td><td>OD-Sca</td><td>Page 1</td></tr></table>				Type	Spacing	Time W	Noise	TN-Sca	ΔOD-Sca	OD-Sca	Page 1
Type	Spacing	Time W	Noise								
TN-Sca	ΔOD-Sca	OD-Sca	Page 1								

Figure 43: B: Measurement parameter menu (page 2)

With the 'Type' function it is possible to select among different types of analysis. This can be Michaelis constant, 1st order rate constant, inhibition constant, pH-dependence and other user defined types of analysis.

The four subroutines of the MICHKIN software exchange the measurement parameters, the syringe parameters as well as flags, counters, and data by means of the COMMON data block. The variables of the COMMON data block are listed in Appendix C.

8.4.2.2 Measure Subroutine

The main functions of the Michaelis measure subroutine are acquisition of photometer data, storage of results and calculation of the kinetic parameters K_m and V_{max} . For this purpose the subroutine has to be able to control both the photometer and the Assayomate.

The syringe control menu is used to move the syringes under manual control. This menu offers the functions fill, empty, suck in, give out, give back and change amount, described in the alphabetical list of functions in Appendix D. The syringes are activated one after the other by pressing softkey #8 in this menu.

An assay is mixed and measured by activating the single assay function in the Michaelis menu. After the softkey 'Single Assay' has been pressed, the user is asked to enter the substrate and the enzyme concentrations. If the entered concentrations are valid, the assay is mixed and the photometer starts measuring. The optical density is plotted on the screen as a function of time, then the derivative, the linearity and the noise are printed.

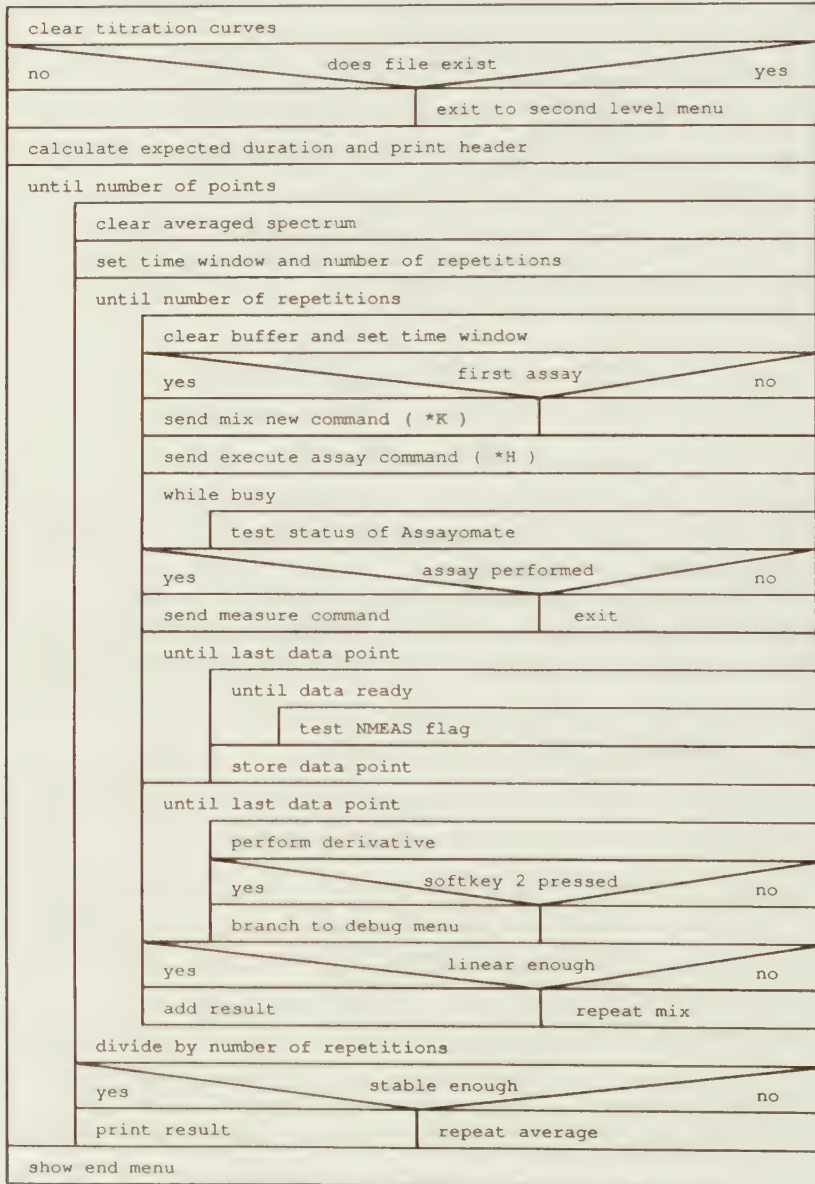


Figure 44: Nassi Shneiderman diagram of MICHKIN data acquisition.

Starting from the same menu, the Michaelis constant together with the maximal velocity is determined by pressing the 'Km meas' softkey. The user has to enter the name of the data file e.g. 'TEST A1', and the enzyme concentration of the stock solution. Then a single assay is mixed and the measured turnover is compared with the selected activity. Depending on the result of this test, the amount of enzyme in the assay is increased or decreased and the assay is repeated. If the turnover number is in the correct range, the header is printed and assays with different substrate concentrations are mixed and measured (see Figure 44). After the last of these assays, the program proceeds to the end menu. In this menu one or several averages (of assays) can be repeated or the result can be stored immediately. Then the kinetic parameters are calculated from a linear regression in a double reciprocal transformation and printed together with the correlation coefficient.

8.4.2.3 Test Subroutine

The test subroutine has three major functions. In the concentration menu the concentration and spectra of substrate and product can be measured. The manual assay menu is used to test assay conditions for new substrates or enzymes. In the serial assay menu, a set of assays can be performed on a large number of probes, either manually or automatically by means of an autosampler.

8.4.2.4 Data Transfer Subroutine

The main function of this subroutine is the transmission of data via RS-232 interface to a host computer. Data files that have been stored on disc are read in and sent to the host. The data transfer is controlled by this subroutine if the softkey 'Master' is selected in the data transfer menu. The data transfer has to be controlled by the host computer if the softkey 'Slave' is selected (see Data Transfer Subroutine of BIRATE software). In addition to data transfer this subroutine is responsible for editing of result files, and plotting of data on an external plotter.

8.5 Test of Assayomate

Before the Assayomate could be used to determine kinetic constants, it had to be tested thoroughly. Among the problems that had to be solved during the test phase were : unstable temperature, insufficient mixing of assays, and bleaching of substrate (photooxidation) by ultraviolet light. Comparison of the results obtained with the Assayomate to results published in literature and determined by manual methods proved the reliability of the new method. The tests which are described in the following paragraphs, summarize the performance of the Assayomate in the configuration that was used for the determination of all kinetic constants in the Biochemical Part.

a) Mixing efficiency and volume delivery

Volume delivery has a relative error of less than 2%. Very small volumes are subject to larger errors. The smallest volume that can be delivered is 0.7 μl , the accuracy is $\pm 0.1 \mu\text{l}$ or $\pm 15\%$ in this case. Immediately before the measurement three solutions are mixed together. A passive mixer provided insufficient mixing for substrate to buffer ratios lower than 1:50. For this reason an active mixer was designed. The design of the stirring chamber allows for homogeneous mixing of solutions differing in volume by 1:1000 within a very short time. (less than 0.3 seconds).

b) Temperature stability

In a first configuration only the stock syringes and the cuvette holder were thermostated. This was insufficient as mixing increased the temperature of the solution by up to 1°C . In the present configuration the mixing chamber as well as the tubings are thermostated. This is sufficient to control the temperature to an accuracy of $\pm 0.2^{\circ}\text{C}$ over the entire temperature range (4°C to 60°C).

c) Blank reaction and noise

Substrates of many enzyme reactions convert spontaneously to the products, but at a very slow rate. However, when the spontaneous oxidation of reduced cytochrome c was measured in the HP 8451 spectrophotometer, the rate of oxidation was much too large : Reduced cytochrome c exposed to the full spectrum of the deuterium lamp of the HP 8451 was photooxidized at a rate of 0.8% per second. Removal of light with wavelengths lower than 250 nm prevented photooxidation.

After the ultraviolet light had been removed, the spontaneous oxidation was within the range of published values. The residual changes in optical density were randomly distributed around zero (Figure 45 B, dotted lines). The absolute noise was less than ± 0.00002 OD per second at all substrate concentrations. For most assay conditions this was a relative noise of less than 1% (Figure 45 A, solid line). These signals were mostly due to electronic noise and due to slightly inhomogeneous mixtures.

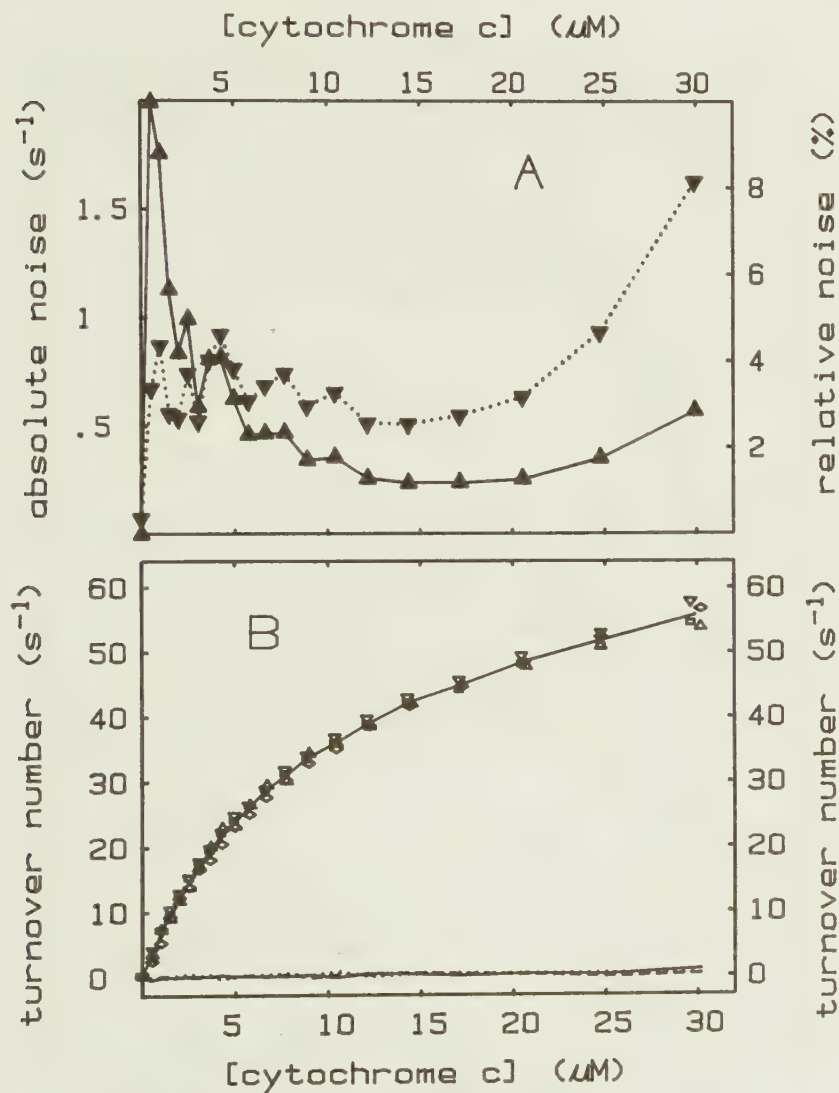


Figure 45: Reproducibility and noise of assay series

Panel A : (∇ , $\cdots$) absolute noise in $\Delta OD \times 10'000 / \text{sec}$ at 550 nm. This was calculated from six independent measurements with 4 repetitions each (total 480 assays), without additional repetitions at low substrate concentrations. (Δ , —) relative noise in %.

Panel B : (Δ , ∇ , $\square$, $\square$) four series of assays measured under identical conditions. (—) average of these four series. Blank reaction of reduced cytochrome c without oxidase but otherwise same conditions ($\cdots$, three determinations).

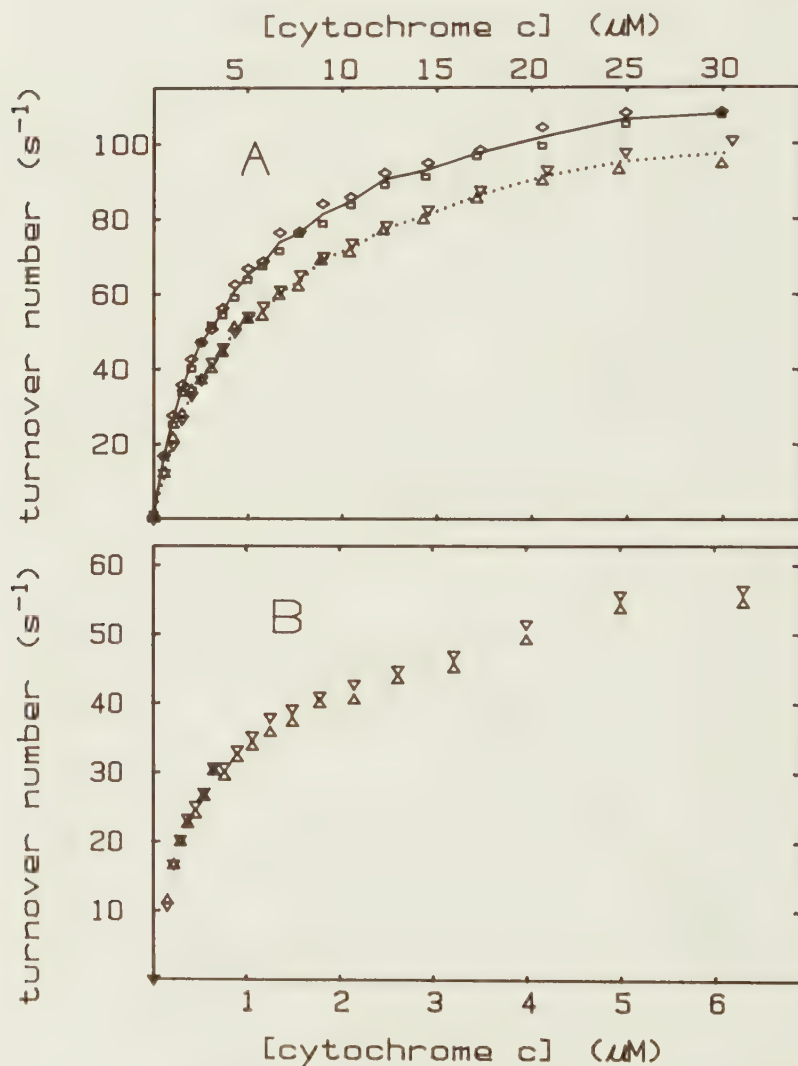


Figure 46: Reproducibility at different enzyme concentration and at different wavelengths

Panel A : (□, —) steady-state activity (s⁻¹) of oxidase (2 nM in the assay). (Δ, ·····) steady-state activity of oxidase (4 nM in the assay).

Panel B : (Δ) steady-state activity of oxidase measured at 550 nm. (▽) steady-state activity of oxidase measured at 418 nm.

d) Reproducibility of series

Several series of assays performed under identical conditions were averaged and the mean root square for all substrate concentrations was determined (Figure 45 B, solid line). The relative error was 10% for the lowest concentrations. Higher concentrations were measured with an error of less than 3% (Figure 45 A, solid line). The reproducibility of kinetic parameters was tested with different concentrations of enzyme. Doubling the oxidase resulted in a 90% increase of cytochrome c turnover (0.00285 instead of 0.00150 s⁻¹ Figure 46 A). It is known that overall activity of oxidase does not increase proportionate to the enzyme concentration! The Michaelis constant was not affected by changes of enzyme concentration.

Monitoring the turnover of substrate at different wavelengths (418 and 550 nm) gave identical TN_{max} and K_m as long as the optical density did not exceed 1.5 AU at one of the two wavelengths (Figure 46 B and Figure 47 G).

e) Reliability of results

The most important test of any new technique consists in the comparison of the results obtained by the new technique with results obtained by other methods. For that purpose, the kinetic constants for a variety of enzymes have been determined with the Assayomate. Among the best characterized enzymes measured were alcohol dehydrogenase and aspartate aminotransferase. For both enzymes a large number of kinetic constants have been published in the biochemical literature. The results obtained by the Assayomate were well within the bandwidth of the published constants. Manual determination of kinetic constants with the same lot of enzyme using the same buffer conditions resulted in the same kinetic constants. But the reproducibility of the results was much better than for manual determinations.

8.6 Sample Result

Before the photometer, the computer and the Assayomate are switched on, they have to be connected by the IEC bus. The photometer and the Assayomate are then switched on, they perform the initiation simultaneously (see main program page 161 and system software page 156). Starting from the main menu, a measurement parameter file has to be read in. Then the concentration of the substrate is determined with the test subroutine. When the test subroutine is executed for the first time, syringe parameters are loaded into the Assayomate memory. But before the photometer is able to perform the first measurement, a 'Reference Measurement' has to be performed. To do that, buffer is sucked into syringe 1 (either with functions from Assayomate keyboard or with the syringe control menu). The reference measurement can be executed from the test assay menu (see 'Michkin User Manual'. After that the substrate concentration is measured in the concentration menu. Then the concentration of the substrate is determined with the test subroutine.

Printouts of results from the determination of kinetic constants can be generated in two modes: If the print flag is on, a full printout is generated a) - h), otherwise only a) and h) are printed.

A typical full length printout consists of 8 parts.

- a) line 1: header with serial number of experiment (B5) and file name (HORSCC)
line 2: date, disc volume name (Modcyt)
- b) This is a list of important measuring parameters
line 1: type of measurement (Michaelis constant initial velocity)
line 2-4: time window at lowest and highest substrate concentration
line 5: measuring interval and integration time for each measurement (integration time $\leq$ measuring interval)
line 6: noise, determines the standard deviation within which single measurements must fall so that average is calculated.
line 7-9: two monitoring wavelength ranges and wavelength range for normalization (no absorption change during reaction in this range)
line 10-11: concentration of enzyme in stock and in assay
line 12-13: concentration of substrate in stock and highest substrate concentration in assay
line 14: number of different substrate concentrations (points) and number of repetitions for single substrate concentration.
line 15-16: extinction coefficient for wavelength ranges on line 7-8. Negative sign indicates decreasing absorption during reaction.
line 17: volume of assay (sum of buffer, enzyme and substrate)
- c) The change of optical density per second is printed for both wavelength ranges. The third column lists the absolute standard deviations of the averaged points of range 1.
- d) Plot showing the derivative of the time course of the optical density versus time ($\Delta OD/sec.$) for all data points. Horizontal straight lines in the ideal case. This plot gives a visual impression of the time independence of the initial velocities.
- e) Header a) is repeated and two lines of comment are printed.
- f) Table of the turnover numbers at different substrate concentrations used for the generation of plot g)
- h) Result of a linear regression of the kinetic data in a double reciprocal transformation.

The accuracy of kinetic constants is increased when the measurements are corrected for a blank reaction. Such a blank can be measured by setting the enzyme stock concentration to zero. It is then stored as $\Delta OD/sec.$ table on the system disc and subtracted from all subsequent measurements.

Figure 47: Sample Michaelis constant determination

- A: Header with file name, name of disc volume, and date of measurement.
- B: Measure parameters (the function of the measurement parameters is explained in the Main Program Section).
- C: First and third column : decrease of absorption at 548 - 552 nm ($\Delta OD/sec.$) and standard deviation of three determinations. Second column : decrease of absorption at 416 / 420 nm ($\Delta OD/sec.$). Both ranges are normalized to the absorption at 580 - 586 nm.
- D: Plot of the derivative of absorption as a function of time ($\Delta OD/sec.$) at 20 different substrate concentrations.
- E: Header for second page with 2 lines of comment.
- F: Turnover numbers at 20 different substrate concentration calculated from data of Table C with the difference extinction coefficients and the enzyme concentration given in B. First column : Point no., second column : turnover number determined from the absorption change at 548 to 552 nm, third column : turnover number determined from the absorption change at 416 to 420 nm, fourth column : substrate concentrations in (μM).
- G: Plot of turnover number versus substrate concentration using the values from Table F. Δ , 548 to 552 nm; ∇ , 416 to 420 nm.
- H: Maximal velocity (or turnover number) in 1/s and Michaelis constant in μM calculated from linear regression of double reciprocal transformation of data from Table F. Third line : correlation coefficient of linear regression.

```
Measurement :  HORSCC  B  5
6. Nov. 86   Volume :  Modcyt
```

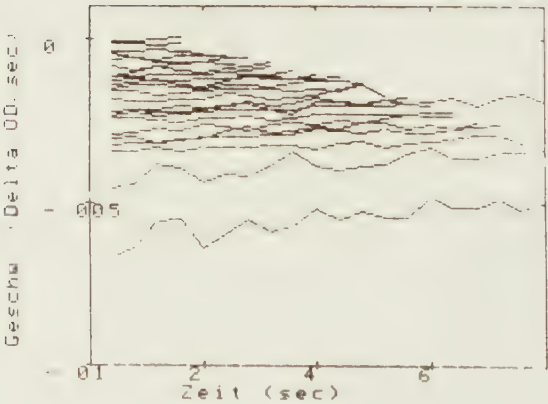
A

```
Time window :
at [S]min      .4 to      1.6 sec
at [S]max      3.2 to      8  sec
Interval       .4      Integration .4
Maximal Noise                5 %
λ-range 1 from      549 to 552 nm
λ-range 2 from      418 to 420 nm
Internal ref. from 580 to 586 nm
[cytochrome oxidase] : 30 nm
conc. in assay       : 3 nm
[cytochrome c]       : 250 μM
Max. conc. in assay  : 30 μM
No. of points      20      Rep.    3
Delta epsilon 1 : -16.2 1/(mM cm)
Delta epsilon 2 : -43.1 1/(mM cm)
Assay volume      0.4 ml
```

B

No.	Range 1	Range 2	Noise
0	-.000029	-.000114	.000014
1	-.000153	-.000178	.000024
2	-.000399	-.001021	.000012
3	-.000559	-.001460	.000023
4	-.000655	-.001778	.000028
5	-.000797	-.002117	.000031
6	-.000914	-.002565	.000029
7	-.001067	-.002885	.000036
8	-.001206	-.003216	.000051
9	-.001281	-.003468	.000013
10	-.001402	-.003778	.000031
11	-.001550	-.004213	.000045
12	-.001706	-.004633	.000059
13	-.001937	-.005262	.000053
14	-.002094	-.005669	.000037
15	-.002317	-.006200	.000058
16	-.002529	-.006613	.000042
17	-.002700	-.006712	.000055
18	-.002817	-.005955	.000054
19	-.003169	-.004821	.000146
20	-.003628	-.003112	.000150

C



D

Figure 47 : Sample Michaelis constant determination (2)

chapter 9

THE MICHFIT SOFTWARE

9.1 Introduction

When large amounts of data have been measured and stored in digital form it is advantageous to use a computer for processing of the data. Typically, spreadsheets are used for the purpose of calculating sums of arrays and to plot the data. Several commercial programs could have been used for this task (e.g. Lotus, dBasell, Visi Calc). But most of these programs are not able to automatically average data from several files and to extract titration curves out of series of difference spectra. They are quite slow if very large amounts of data have to be processed.

It was much more difficult to find an appropriate software for direct curve fitting. There have been statistical packages available on mainframes, but not for the analysis of biphasic binding processes. Moreover, no software was available to transfer data from a personal computer to a mainframe.

Several statistical packages that are available on small computers have been examined. All these programs were not very flexible and could not be implemented into a user friendly environment. Most graphical software and most of the spreadsheets are designed as 'stand alone' programs. The file structure of these programs is sometimes not laid open. In no case import of external files was thoroughly explained in the handbooks.

Integration of fitting programs with spreadsheet functions required the development of a new software. Several subroutines were already available in BASIC. For that reason BASIC was selected as the high level language. The system software was written using a 8088 Macro Assembler. The programs were used on a HP-150 personal computer (8088 CPU, 8 MHz).

9.1.1 Aims

The software described in this chapter should be able to process the files generated by the MICHKIN program, by the BISTRATE program, by the HP 8451 spectrophotometer software, and optionally, by other software.

It should perform linear regressions and direct curve fits. The user should be able to check the quality of the fits by visual inspection of data points and a fitted curve in transformed plots (e.g. double reciprocal, Eadie Hofstee). Averaging of several sets of experimental data measured under identical conditions should be easy and fast. It should be possible to use data sets as operands in mathematical operations.

The software should be clearly structured (menu oriented) and easily understandable for users with a chemical, biochemical, or biological background. All commands should

be executed using a touchscreen or a mouse. The program should run with no need for the user to know DOS-commands.

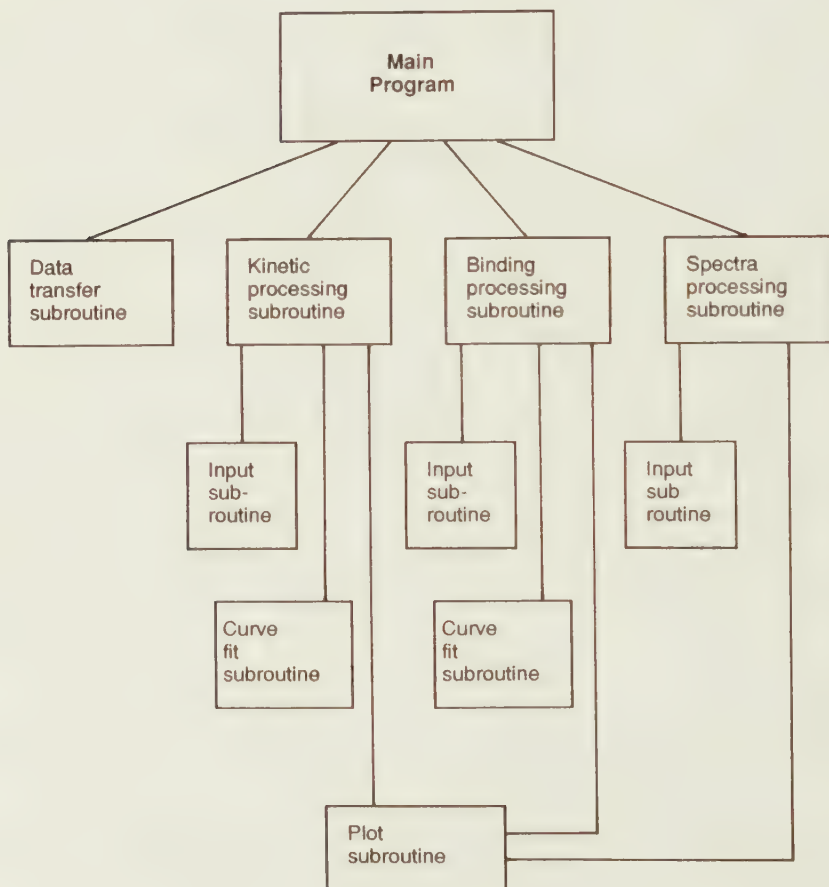


Figure 48: Survey of MICHFIT software

9.2 System Software

The high-level languages Microsoft compiled BASIC or Microsoft PASCAL implement only a small set of commands. All other commands or functions have to be implemented with CALLS to assembly language subroutines that are linked to the object modules. Among the functions that had to be implemented are:

- graphic functions
- touchscreen functions
- access to system clock
- read disc directories
- input-output routines (RS-232)
- multiple screens

Some functions can be executed by printing ESCAPE sequences to the terminal, but at a very slow speed. The HP-150 offers a built-in library of functions accessible by assembly language calls, the AGIOS functions.

The alphanumeric and graphics input-output subsystem (AGIOS) is a set of system functions that are used to access the alphanumeric and graphic intrinsics of the HP-150. These function calls perform text and graphics mode operations on the display, control the keyboard, define application softkeys and perform touchscreen operations.

AGIOS provides a five times faster access to the terminal features than comparable escape sequences. In addition AGIOS provides functions for transferring a block of data to the display.

Each AIOS or GIOS function is identified by a 16 bit function code. The function code and additional parameters have to be stored in a command buffer (see Appendix C). Then the processor registers have to be set up, and the function is executed by an interrupt to the operating system.

The HP-150 provides a set of data communication I/O functions available in MS-DOS. The 'data comm' functions are accessed in the same way as the AGIOS calls (by a command buffer).

The performance of the MICHFIT software was considerably improved by two routines that store and recall the content of the alphanumeric screen from a mass storage medium :

The STOSCR (#) function is used to store the contents of the alphanumeric screen to a random access file on a mass storage medium, preferably a RAM disc or a hard disc. With the RECSCR (#) function stored screens are recalled from a random access file that has already been opened. Only the first 21 lines of the screen are stored. The only limitation to the number of screens that can be stored is the storage space and the speed of access.

9.3 The MICHFIT User Software

9.3.1 Principle of Operation

The MS-DOS operating system (version 1 - 3) is a single task single user system. The file access is quite slow, especially to a hard disc when many files have been created and deleted. The reason is the lack of a reorganization task for a hard disc and a poor organization of the directory:

The quickest access is achieved to files that are created shortly after the initiation of the disc and that are never deleted. As the program had to be split into several subprograms that are loaded into memory one after the other, optimization of disc access was essential.

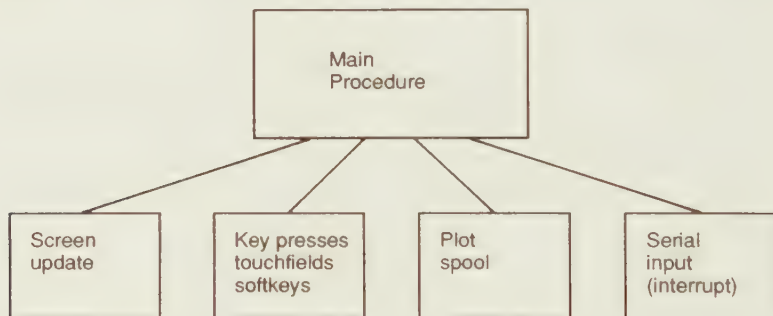


Figure 49: Operation of MICHFIT user software

The subprograms use a main procedure that activates the different subfunctions of the program one after the other (Figure 49). Single tasks of these subfunctions are very short so that a pseudo real time performance is achieved. The subfunctions are:

- screen update
- keyboard input
- background plot (from file)
- serial interface input (to file)

Before a menu is left, the content of the alphanumeric screen is stored in a file. When the same menu is again selected, the screen is recalled from the file. Then, the new functions of the softkeys are labelled. After these two tasks control is given back to the main procedure. This organization is especially useful if another subprogram has to be loaded. In this case the new menu is displayed before the actual program is ready. The user is able to study the menu while the computer is still working in the background. Key presses are saved and executed as soon as the overlay is ready (max. 5 seconds). The subroutine

checks the fields of the masks (20) and changes them if necessary. As the menus work on different tasks, modifications of the content of one menu has no effect on other menus.

Key presses can be detected in two modes:

- command mode
- interpreter mode

In the command mode each byte is executed immediately. In the interpreter mode the execution starts only after a (cr) byte has been received (see Interpreter Subsection).

9.3.2 Main Program

The MICHFIT software processes files generated by the BITRATE, the MICHKIN, and the HP 8451 spectrophotometer software. Data from other sources can be received via a RS-232 interface. It was written around a workfile, an array of 100 records stored in two files on the hard disc. These records are used as operands of mathematical operations or as sources for direct curve fits and graphical presentations. The main program has seven main functions:

- initialize COMMON data area
- read directories
- edit menu masks
- edit system files
- activate workfiles
- call subprograms

The MICHFIT software is started by the main program (HA.EXE). The user has to select one account (1-20) and has to enter a six character password. If the password is entered correctly, the initiation routine is started. First, several parameters defining the configuration are read from the main system file DEFAULT.HSY. Second, the common variable are initialized and the default values of the plotsize are read from the system file USERXY.HSY. Third, all data files on the discs a:, b:, and c: that belong to the user are read, sorted according to file type and stored in a random access file on the fastest mass storage device. After the initiation, the main menu is displayed. The main menu offers functions for modification of system files and screen masks.

Moreover, it is used to initialize the system files and the workfiles for new users. Most of the time, the main menu is used to select among 4 different types of subroutines:

- processing of kinetic data
- processing of binding data
- processing of spectra
- data transfer

The first three data processing subroutines perform very similar tasks on different types of data (see Figure 48). The only differences are the output transformations. The spectra processing subprogram uses a different workfile than the other two subroutines. In the case of spectra only y-values are stored, the x-values have to be spaced evenly so that only x-min, x-max and delta x have to be stored.

Touching one of the three large fields in the main menu starts the respective data processing subroutine. But before control is passed to the subroutine the main program searches the system files and the screen file on the a: disc. If these files are not found, the default system files are copied to the user system files. Then a file containing the status at the last exit from the data processing subroutine is read. If it is not found, the default status initialized by the main program remains in effect. After these tasks, the main routine of the data processing subroutine receives control. It displays the input menu and starts checking the keyboard, the touchscreen and the output devices. In order to speed up the initiation procedure, reading of the header of the workfile is performed as a background job.

The MICHFIT software is composed of several subroutines that are loaded into memory according to commands of the user. Each of these subroutines uses a set of local variables.

Global variables are passed between the subroutines by means of a **common data block**. The entries in this block are listed in Appendix C.

9.3.3 Data Processing

The 8088 and the 8086 processors support data blocks up to a size of 64 kBytes only. Thus it was not possible to hold the header and the data of more than 15 spectra simultaneously in memory. But for the evaluation of titration curves from difference spectra it was necessary to hold simultaneously up to 80 spectra in memory.

For this reason the spectra are stored to a random access file. The header information is stored on disc too, but in a separate random access file. For the sake of a good performance it was necessary to hold a part of the header information constantly in memory, the rest of the header and the data are read in from the files only when they are actually needed. With this approach the total number of data sets is only limited by the capacity of the mass storage medium. Another advantage of this approach is the good security of the data, because all changes are immediately written to the disc. Power failures never cause loss of data.

The data processing subroutine was designed so that the user can access the information stored in a special file, called the *workfile* in the subsequent text. One entry, a record, of the workfile contains the header

- 1 a string with directory entry, enzyme name, ligand name, date
- 2 8 integer variables
- 3 8 real variables

and the data of a spectrum or a titration curve. Due to the limited memory and disc space available, it was necessary to limit the number of data points to 321.

It is possible to recall one of the six menus : INPUT, OUTPUT, CONTENT, PLOTSIZE, DISPLAY, and FIT from anywhere in the program using a fixed set of softkeys. These softkeys are constantly checked in the command mode.

9.3.3.1 User interface

From the view point of the user the workfile is the inside of the program. The content of this workfile can be inspected and modified by means of the CONTENT menu. The INPUT menu is responsible for storing of data in the workfile. With the OUTPUT menu it is possible to select up to ten data sets (records) and to send them to an output destination (e.g. display, plotter).

a) CONTENT menu

The screen of the content menu shows the headers of 17 records out of the workfile.

No.	Name	Y-min.	Y-max.	X-min.	X-max.	Step
1	title	+0.001	122	390.0	450.0	0.5
1	2	3	4	5	6	7

The first entry is the record number, then follows the name of the spectrum or titration curve. In addition to that, the minimal and the maximal x and y values are displayed. The next entry is the step size between the digitalized values. The entries in the content menu are slightly different in the binding and kinetic data processing subroutines.

The softkeys 'former page' and 'next page' are used to change the different pages of the workfile. The rarely needed entries are displayed by the 'status record' function in the second set of softkeys. All other functions in the content menu are used to modify the workfile (copy record, erase record, new name, edit data). Excessively noisy spectra can be smoothed with the 'smooth data' function. Arithmetical operations with records are performed using the interpreter (see below) or the 'arithm. operation' function.

The 'swap workfile' function is used to copy the entire workfile (header and data) to a data disc and to overwrite the internal workfile with another workfile.

b) INPUT menu

The input menu is responsible for all inputs coming from outside the workfile. The data source touchfield of the input menu switches among several devices capable of providing data:

disc drive A, disc drive B, disc drive C, and the keyboard.

If the data source is a disc drive, the file type has to be selected with the touchfield on the same line. Before an input is actually executed the destination record in the workfile have to be entered. Then the input is started by depressing the softkey 'Execute input'. Depending on the setting of the 'no. of files' 'no. to average' field, one or more fields in the stored files area have to be touched. After that, the file names and the other parameters are passed in the common data block to the input subroutine. This subroutine actually executes the commands (see the description of the MICHKIN software, the BITRATE software, or the HP 8451 for the description of the file format).

If a keyboard input has been selected, the header information, the integer and the real variables together with the data points have to be entered one after the other. If the

input has been successfully terminated the data processing subroutine is reactivated and displays the output menu.

The 'copy file', the 'rename file', and the 'erase file' function are used to move and process the external files. The 'show file' is used to display the contents of a file on the screen without actually reading the contents of this file into the workspace. The 'display directory' function is used to display all files as they have been stored on the disc whereas within the stored files area only files of one file type that belong to the actual user are displayed. The files are ordered by their extension, the first character defines the file type, e.g. P = plot file, S = spectra, K = kinetic file, D = difference spectra file, X = X-Y data file. The next two characters are the initials of the user, e.g. BM for Bruno Michel. Thus the HP 8451 spectrum 'SPECTR1' of the user 'BM' is stored as the file 'SPECTR1.SBM'.

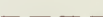
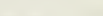
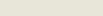
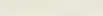
1		11		21		31	x
2		12		22		32	+
3		13		23		33	*
4		14		24		34	.
5		15		25		35	■
6		16		26		36	○
7		17		27		37	¤
8		18		28		38	%
9		19		29		39	\$
10		20		30		40	&

Figure 50: Symbols and lines defined in output menu

c) OUTPUT menu

The output menu is responsible for all outputs from the workfile to other subroutines or devices. The destinations are Display, Plotter, Printer, Spool file, Plot file, or an External subroutine. The data is output directly or a transformation is performed on the data before it is output. This transformation is selected by the 'mode' field, the field to the left of the 'destination' field. The transformations vary among the kinetic, binding, and spectra data processing subroutines (Table 13).

The lower half of the output menu is used to select the records that are output. The number of the selected curves is determined by the entry in field 'No. of curves', it can range from zero to ten. In this lower half the symbol of the curve (1 - 40) and the pen (0 - 8) of the respective curve is selected (Figure 50). In addition to that, the two window fields

No.	Spectra	Kinetic	Binding
1	no transform.	no transform.	no transform.
2	1st derivative	double reciprocal	double reciprocal
3	2nd derivative	Eadie Hofstee	Eadie Scatchard
4	transmission	Hanes Wolfe	normalized plot
5	ln Y	Hill	Hill
6	interpreter	interpreter	interpreter

Table 13: Transformations

are used to specify the start and the stop of the curve output. The values entered in the window fields have to be within the range that is actually stored.

9.3.3.2 Interpreter

The interpreter can be activated in all data processing subroutines and in the kinetic curve fitting subroutine. It accepts a string of commands terminated with a carriage return. The commands can be mathematical operations with numbers and/or spectra, titration curves for example :

$$Y11 = (Y2 + Y3) / (10 \wedge Y1 * Y2 + Y3) - Y4$$

Y1 means that the Y-values of the spectrum 1 are taken. The operations that can be performed are :

L (logarithm), ^ (exponent), *, /, D (derivate), +, -.

At the beginning of each operation, the operands are loaded into the accumulators. In case one operand is a number and the other operand is a two dimensional vector of n elements, the number is copied n times. If both accumulators hold two dimensional vectors of the same size the operation can be performed instantly. If the two vectors are of different size or if they use a different spacing, the vector to the left has to be adjusted to the other vector, this is done by the operation adjust procedure. This procedure takes the X-elements of the right vector and interpolates the data from the Y-elements of the left vector. All points where the vectors don't overlap are removed. If the resulting number of points is smaller than three the operation is aborted.

The interpreter takes the content between the highest priority parentheses, in our example 'Y2 + Y3', and locates the operator, then the left and right accumulator are loaded with the Y-values of the curve 2 and 3. Then the operation is performed and the intermediate result is stored in the record Z9, the string (Y2 + Y3) is then replaced by Z9.

$$Y11 + Z9 / (10 \wedge Y1 * Y2 + Y3) - Y3$$

Next the content of other parentheses with the same priority is solved :

$$10 \wedge Y1 * Y2 + Y3$$

Here, the highest priority operation is located (^) and the accumulators are loaded:

(left accumulator n times 10, right accumulator Y-values of curve 1), and the operation executed, after the operation the commands becomes

$Z8 * Y2 + Y3$

Next the multiplication is executed leaving the command as follows:

$Z8 + Y3$

Finally, the addition is performed and the result is copied to Z8.

After the content of the parentheses has been solved and the intermediate result is stored in the Z8 buffer the command string appears as follows:

$Y11 = Z9 / Z8 - Y3$.

The division is executed, the result stored in Z9, then the subtraction is executed and the result is again stored in buffer Z9. The Y-values of the record 11 are loaded with the content of the Z9 buffer and the curve is stored on disc.

One of the advantages of this interpreter is its high flexibility. It can use an unlimited number of source records and an unlimited number of parentheses, the only limitation being the stack size of unresolved parentheses of the same priority (max. 9). Moreover the design of an interpreter was a prerequisite for interpreting models in the fit routine and for the interpretive command language.

Before the string is sent to the operation interpreter a command interpreter tries to find executable commands in the string. This is used to define a data base language interpreter. This interpreter accepts files as input source. For this reason it is able to interpret and execute stored programs.

9.3.3.3 The Plot Files

Plot files have two functions, first they store all commands that are necessary to produce a plot (size, frame, annotate, draw). Second, they store all data (spectra, titration curves) so that they can be used in the workfile like other data once the plot file has been read in.

This approach is most flexible because data curves (up to 10) can be stored together in a file. The plot can be documented with 15 lines, this allows to process large numbers of files without complicated 8 character file names. It is easy to check the content of plot files because they can be read in quickly using the input menu.

A plot file is composed of several parts : The first entry in the plot file is the encoded version number of the software by which the file was generated. In the next block the axes, the position of the plot, and the positions of the labels together with other values are stored. Then a pointer counting the number of draw points is stored. If this pointer is not zero, a series of n entries with three integers is written to the disc (see Plot Functions). Next the title, the labels of the axes, and the annotation labels are stored. The header and the data of n records are stored in an encoded format at the end of the plot file.

9.3.4 Graphical Presentation of Data

The major function of the plot subroutine is the graphical presentation of data on the screen or plotter. The data can be printed in tables on the printer. It is called by the spectra, binding, and kinetic data processing subroutines. After the task of this subroutine (the plot) has been finished, control is given back to the calling subroutine. It is able to process ten curves of maximal 321 data points each. The number, symbols, and pens of the curves are defined in the output menu and passed to this subroutine by means of the common data block. In addition to the curves, several other parameters are passed to this subroutine:

autoscale	on/off	line interpolate	on/off
zoom	on/off	display	positive/negative

If the autoscale flag is 'ON', the axis of the display or plot are set so that all data points appear within the defined area. In addition to the minimal and maximal values, the steps for numbering the axis are set to proper values.

If the zoom flag is 'ON', the plot is enlarged, so that it occupies the entire screen but without changing the proportions. In the 'zoom OFF' mode the screen is treated as an A4 format, and the graph is drawn as it would appear on an A4 sheet of paper. The 'positive/negative' switch selects between light symbols on dark screen or dark symbols on light screen. The line interpolate flag is used for lines only. If this flag is set, more data points between the stored data points are generated, this gives the curve a smooth appearance on the screen and on the plotter.

9.3.4.1 The Output Devices

With the output destination touchfield in the plotsize menu or the hardcopy submenu of the display menu, it is possible to select different output devices. Each of these options activates a different subroutine which is responsible to channel the output to the respective device.

a) Display

The plot appears on the screen in two modes. In the 'zoom on' mode the plot is enlarged so that it occupies the entire screen. In the 'zoom off' mode the screen is treated as an A4 sheet and the plot is shown proportionate to the size of the sheet.

The 'lock display' flag is used to protect areas of the graphic display against overwriting. If a new plot occupies the same window of the display it is not executed and the message 'display locked' is displayed. The 'lock display' flag is cleared with the 'unlock display' function. Pressing this softkey immediately draws the plot to the protected window. If the 'lock display' flag is not set in the 'zoom off' state, the plot is removed from the screen each time the plot subroutine is left. Plotting, spooling, or filing automatically sets this flag.

b) Plotter

The size of the plot frame and all other parameters have to be set in the plotsize menu. This subroutine has been designed to give an identical picture of the display on the plotter.

c) Printer

This subroutine prints the data points as x- and y-values on the line printer.

d) Spool file

This subroutine is similar to the plot subroutine with the exception that the plot commands are written to the disc. With the 'spool command' softkey in the plotsize menu or the 'spool' touchfield in the other menus the output of the plot spool is controled. The plot spool reads the plot commands from the disc and sends them to the plotter while the user is able to perform other tasks.

e) File (plot file)

Plot files are not generated by the plot subroutine but they can be initiated from the plotsize and the hardcopy menus. The plot files are described in the output menu subsection.

f) Screen dump

The screen dump function is implemented by firmware of the HP-150. This routine prints an identical image of the screen on the printer.

9.3.4.2 Plot Functions

Five types of data visualization are implemented in the plot subroutine:

a) Plot lines

This subroutine connects data points with straight lines. Ten different line types can be selected (symbol 11 - 20, figure 50). If the 'interpolate line' flag is set, more points are generated between data points. A parabola is fitted to the data points in order to generate the intermediate points.

b) Plot symbols

User defined symbols are read from the main system file and plotted at each data point. They are defined separately by each user. They are accessed with symbol types 1 to 10. Another 10 characters out of the ASCII character set are used to be plotted at data points (symbols 41-50).

c) Plot columns

In this mode a column is drawn for each data point. The width of the columns is defined in the plotsize menu (col. w) in percent of the x-size of the plot. The column starts at the x-axis, its height is determined by the y-value of the data point. The columns are filled in 10 different ways (e.g hatch cross hatch).

d) Annotate

A set of softkeys controlling the annotation of the plot is activated with the 'annotate plot' function in the display menu. Up to 15 lines of text are written to the plot by this subroutine. The text can be placed anywhere on the plot using the graphic cursor of the display menu. The position of the text is stored in relative units (1-100%) of the frame. Changing the size of the plot (x- and y-size) or the orientation (position of plot) does not affect the relative position and orientation of the text. The text and the positions of the lines are

edited in the lower half of the plotsize menu. Examples of annotation are found in Figures 3, 4, and 7 and Table 10.

e) Draw

This subroutine draws lines using the graphic cursor. It is activated by the 'draw' softkey in the display menu. Up to 128 data points are entered and edited in the draw submenu of the display menu. In addition to draw lines, it is possible to draw symbols and columns anywhere on the plot. Symbols, lines and columns are selected with the graphic cursor on the 'select symbol' submenu. Examples using the draw subroutine are found in Figures 3, 7 and Table 10.

9.3.4.3 User Interface

With the two menus of the plot subroutine, the DISPLAY menu and the PLOTSIZE menu, all aspects of the plots can be set to meet the demand of the user :

a) DISPLAY menu

Unlike all other menus the display menu uses the graphic screen for the display of data and of the softkey labels. A cursor is used to show the x-and y-values of the data points. In a second mode the cursor is used to draw lines (see Draw), and to put labels to any part of the plot (see Annotate).

b) The PLOTSIZE menu

The plotsize menu is responsible for the size and the orientation of the plot. The display is altered by this menu, because it is designed to show an identical image of the plot (see MICHFIT User Manual). Besides the softkeys a comprehensive set of touchfields modifying each property of the plot is offered :

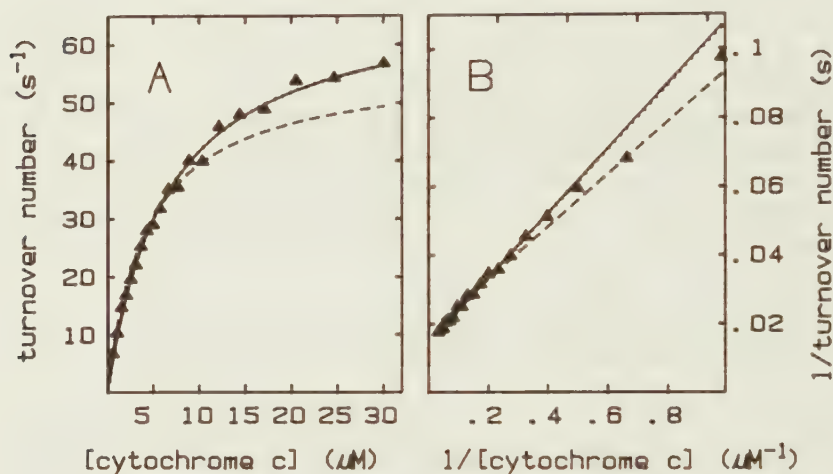
- The size of all labels, their position and their direction can be altered.
- The elements of the axes (ticks, digits, labels) are independently switched on and off for all axes (x-lower,y-left, x-upper, y-right).
- The direction of the plot can be selected (across, along).
- The size of the plot can be selected in cm (x- and y-size).

For a complete description of the plotsize menu see MICHFIT User Manual.

9.3.5 Direct Curve Fitting

Because computers are cheaper and easier to handle, fitting of models to experimental data has become a useful tool for the biochemist. The conventional methods that have been used to analyze the data, the linearization procedures, often led to wrong answers. For example the linear regression to a double reciprocal transformation puts too much weight on the inaccurate data points at low substrate concentration (Figure 51).

Many researchers are prevented from using direct curve fitting programs because they are not familiar with partial derivatives. Another problem is the lack of transparency of the fitting routines, either they provide a meaningful solution or the result can't be used. The user cannot influence the progress of the fit or have one parameter fixed at a defined value.



C : result from weighted direct curve fit

curve fit to record	: 86 Cox VB Mix u=150 LH1	July 26. 87
cytochrome oxidase	: 2.7 nM cytochrome c	max. 30.00 μM
data points used from	: 1 to 20 record with noise	30
fitted model	: Michaelis Menten	
result	: TNmax. = 68.6129 Km = 6.4680 noise	0.98947

D : result from unweighted direct curve fit (equally weighted)

curve fit to record	: 86 Cox VB Mix u=150 LH1	July 26. 87
cytochrome oxidase	: 2.7 nM cytochrome c	max. 30.00 μM
data points used from	: 1 to 20 record with noise	0
fitted model	: Michaelis Menten	
result	: TNmax. = 68.3750 Km = 6.4404 noise	1.03472

E : result from linear regression

curve fit to record	: 86 Cox VB Mix u=150 LH1	July 26. 87
cytochrome oxidase	: 2.7 nM cytochrome c	max. 30.00 μM
data points used from	: 1 to 20 record with noise	0
linear regression	: Lineweaver Burke (double reciprocal)	
result	: TNmax. = 56.4154 Km = 4.3104 correlation	0.99709

Figure 51: Comparison among direct curve fit and linear transformation

Panel A : Velocity v measured at different substrate concentrations with Assayomate and MICHKIN software.

Panel B : Double reciprocal transformation of experimental data ($\blacktriangle$), a simulated curve from the result of the weighted direct curve fit listed in Panel C (—), a simulated curve of an equally weighted direct curve fit (·····), and the optimal linear regression from panel D (-----).

Panel C : Sample output from direct curve fitting routine with weights shown in Figure 53 (record 30) using a simple Michaelis Menten model (Equation 9 on page 146).

Panel D : Sample output from direct curve fitting routine without weights using a simple Michaelis Menten model (equally weighted).

Panel E : Result of linear regression in a double reciprocal transformation.

----Why a new fitting program

Having in mind the drawbacks of both the linearization methods and the gradient and grid search regression programs available, a new fit program was developed. Like the other programs it is started by entering first estimates. But if no good estimates are available it is possible to use a grid search covering the entire meaningful area at the beginning. Then a binary grid search together with a gradient search routine is used to optimize the estimate found by the first grid search.

Several models are contained in the software, but own models can be entered using a standardized command language :

$$R = C1 * S / (C2 + S)$$

This is an example for a simple Michaelis-Menten model (Equation 9) :

R : Dummy argument telling the system to perform a regression.

C1 : First parameter that will be optimized (V_{max})

C2 : Second parameter that will be optimized (K_m)

S : Substrate concentration S1 to Sn of data set

All algebraic operations (L ^ * / + - ()) can be performed either with numbers or two dimensional vectors.

9.3.5.1 Theory of Direct Curve Fit

In all sciences experimental data has to be compared with a theoretical model. To do this, an algebraic equation relates a specified independent (or controlled) variable to an experimentally observed dependent (response) variable. The Michaelis-Menten equation determines the rate v of an enzymeic reaction at a substrate concentration $[S]$:

$$v = \frac{V_{max} * [S]}{K_m + [S]} \quad (10)$$

V_{max} and K_m are constants representing the maximum velocity at saturating substrate concentration and the Michaelis constant, respectively. This model is usually the simplest way of accounting for the kinetic properties of many enzymes. Approximate values for the kinetic constants can be obtained from the visual inspection of the graphical representation of velocity against substrate concentration (Figure 51 A). The velocity measurements are the experimental data and the values for K_m and V_{max} are derived parameters (not directly measured quantities). An alternative and popular graphical approach is to transform mathematically the model equation into a form giving a straight line plot (1,2). The Lineweaver-Burke plot is such a well known linear transformation (Figure 51 B). Graphical linear transformations have the advantage that gross deviations from linearity are immediately noticeable by visual inspection. However, numerical procedures are more appropriate and desirable if a precise estimation of the parameters and the goodness-of-fit is required. A linear transformation distorts the experimental variability leading to biased estimates for the values of the parameters.

Linear and non-linear regression

In general, a linear or non-linear two-parameter model can be expressed by:

$$Y_i = f(\alpha, \beta, X_i) \quad (11)$$

α and β are true but unknown parameters of the model, Y is the experimentally determined dependent variable and X is the error free independent variable for n pairs of data points. However, experimental data rarely conform to a perfect model. In regression analysis this is taken into account by introducing an error term:

$$Y_i = f(\alpha, \beta, X_i) + E_i \quad (12)$$

The E_i term represents a random error component usually assumed to be normally distributed with a mean of zero. The estimates (a, b) of the parameters (α, β) are obtained from the experimental data by minimizing the sum of squares of the residual error terms in the model equation.

$$S(\alpha, \beta) = \sum [Y_i - f(\alpha, \beta, X_i)]^2 \quad (13)$$

Unlike linear regression, the derivation of an optimal set of parameters by non-linear regression is conceptually more difficult. The optimization procedure usually leads to complex algebraic expressions. To overcome this problem several procedures have been described. The most commonly used methods are based on the gradient, search or linearization algorithms. The Gauss-Newton procedure, widely available in statistical software packages, is a method of local linearization around some initial parameter estimate. An iteration procedure attempts to improve the initial estimates in the least squares sense. At each iteration the residual sum of squares is calculated (Equation 13) and compared with the previous estimate. The iteration is terminated when one of several possible stopping criteria is achieved, usually when the largest relative changes of estimates of the sum of squares function fall below a predefined tolerance value. The success of the Gauss-Newton fit depends crucially on the accuracy of the first estimates. This is not a problem for models with two parameters, but the parameters of more complex models are more difficult to estimate. Wrong estimates often lead to impossible solutions or to the capture of the fit routine in local minima.

9.3.5.2 Description of Fit Subroutine

The curve fit routine that was implemented in the MICHFIT software operates in two modes:

- a) as a grid search routine
- b) as a gradient search routine

Central to both modes is a routine that calculates the root mean square of the experimental data when compared with the theoretical model. Before the fit can be executed, a set of first estimates have to be entered. In addition to the estimates the desired absolute accuracy and a factor for the first grid have to be entered for all parameters.

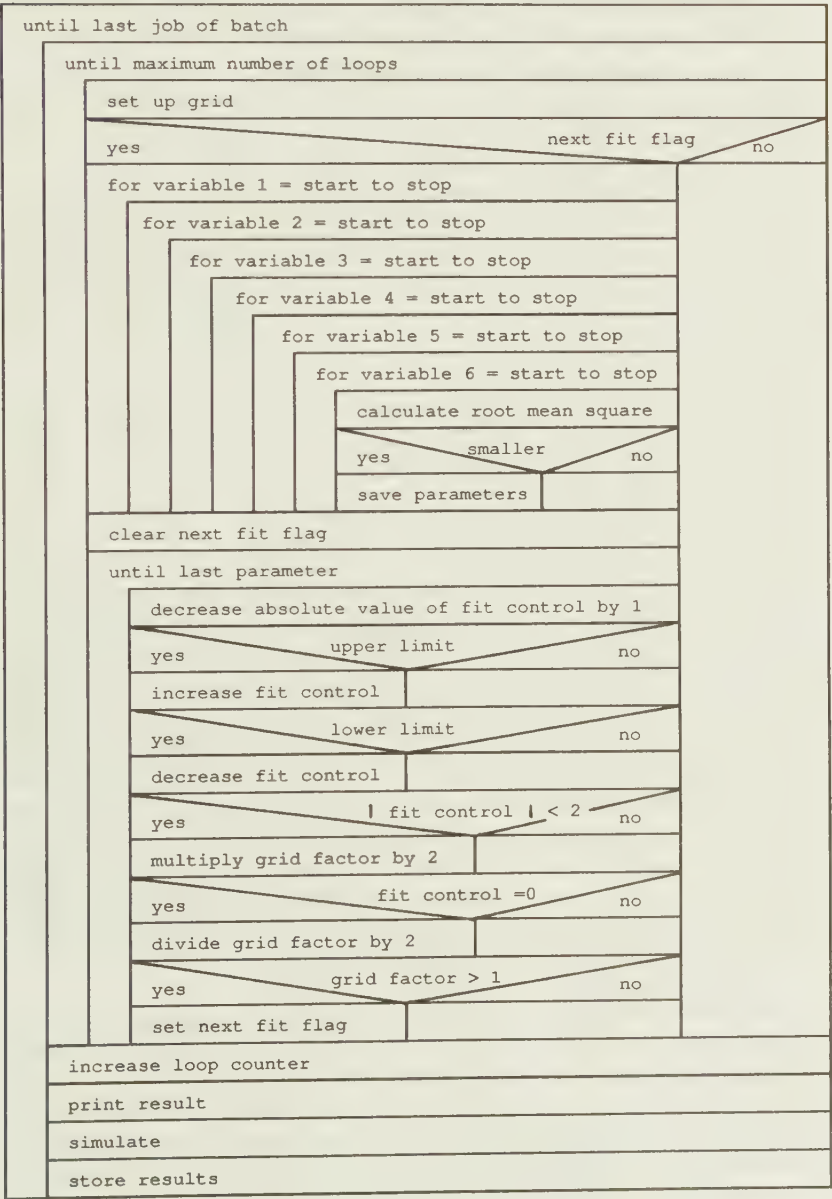


Figure 52: Nassi Shneiderman diagram of direct curve fit routine

The following example shows the input that was made in order to perform the fit in Figure 51 C. The user inputs are printed in *italics* :

Please enter model, record, and points from to : *1,86,1,20*

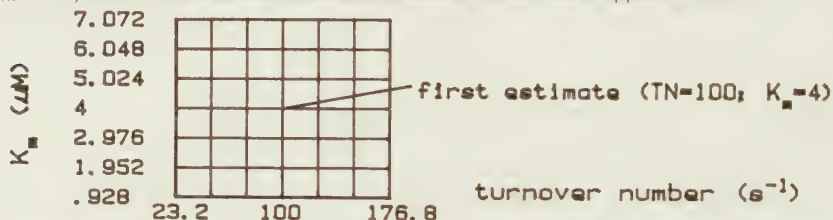
Please enter destination record and max. number of cycles : *90,99*

Please enter record with noise (0=equally weighted) : *30*

Please enter first estimate, grid, and factor for TNmax: *100, 0.1, 256*

Please enter first estimate, grid, and factor for Km : *3, 0.001, 1024*

In this example, the finest grid of the maximal turnover (0.1) is multiplied by 256 for the first search. In most cases the first search is a grid search with 7 values for each variable. In the example the search for the maximal turnover numbers started at 23.2 and stopped at 176.8, and for the Michaelis constants it started at 0.928 and stopped at 7.072.



After all estimates have been entered, a root mean square is calculated for every crossing-point of the grid, and the point with the least square is given back to the main routine. The former estimates are overwritten with the new estimates and the value of the point with the least square is compared with the minima and maxima that have been searched (lower border, upper border). If all values lie within the search range, the factors are divided by two and the next search is started with a grid of only three values for each variable that is necessary for a gradient search. Again, the setup is passed to the root mean square routine. The resulting new estimate can be larger, smaller, or identical when compared with the old estimate. If the new estimate is identical, the factor is again divided by two and another fit is performed until the factor is 1. In the other case a fit is performed using the new estimate. If the second result is again larger or smaller (the same direction only), the factor is multiplied by two and a fit with a larger grid is performed. This is repeated until a new estimate is identical with the old one. With this routine the fit routine is able to escape from local minima and to move quickly to better values for the variables (see Nassi Shneiderman diagram in Figure 52).

The fit routine is terminated when all factors are 1 and identical estimates have been achieved twice in a sequence for all variables. Other reasons for the termination of the fit routine are:

- The convergence of the fit is too slow so that the maximum number of search cycles (entered by the user) is exceeded.
- The 'exit fit' or the 'next fit' function key have been pressed.

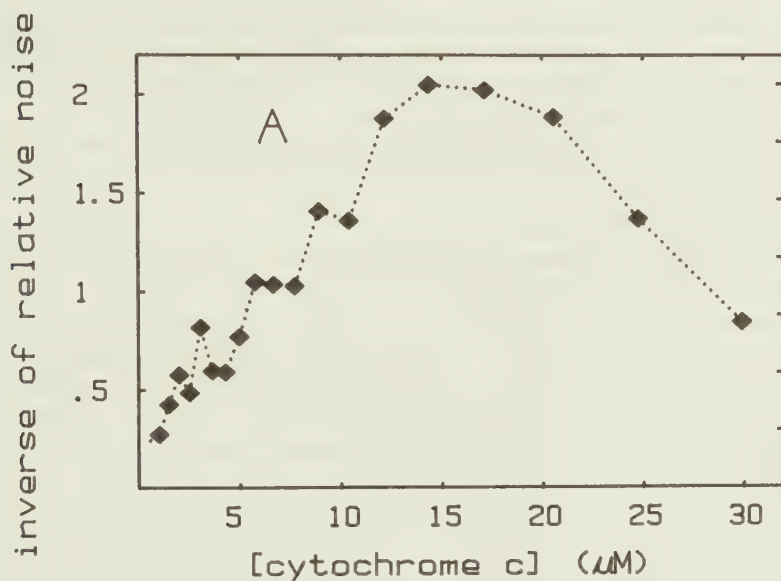


Figure 53: Normalized weights used in direct curve fits

If the fit is terminated due to convergence of all variables, a result is printed and a theoretical curve is calculated from the result. The theoretical curve is stored in a record like the experimental data. It is calculated for the same substrate concentrations as the experimental curve, so that it can be used to calculate residual plots (difference between model and experimental data) and to be plotted together with the data.

This curve fit routine can use an equal weight for all data points or it can use a record as a source for the absolute noise. The weights for the individual data points are then calculated by dividing the experimental data by the absolute noise and normalization of the weights to an average of 1 (Figure 53).

The use of a grid search technique together with a gradient search technique results in a very robust fit routine. It is hardly ever caught in local minima. As it is possible to manually interfere with the fit process, any set of data points can be used.

9.3.5.3 Weighted Least Squares

The results from weighted least squares optimization procedures are much more accurate than linear regressions or unweighted fits. In Figure 51, the result of weighted and unweighted curve fits are plotted in a turnover number versus substrate plot (Panel A) and in a double reciprocal transformation (Panel B) together with a linear regression. It is clearly visible that the linear regression assigns much more weight to the inaccurate first data points (the points with the lowest substrate concentration). The unweighted direct

curve (all points are equally weighted) puts slightly too much weight on the first data points. The Michaelis constant from the weighted and the unweighted (equally weighted) sample fits in Figure 51 are not largely different. Results from fits to models with more parameters, however, would differ much more.

It is therefore strongly recommended to use weighted least squares optimization procedures for analyzing kinetic and binding data.

9.3.5.4 Fits with Interpreter

Novel to direct curve fit programs on personal computers is a feature that is based on the operation interpreter described above.

The interpreter uses the model entered with the function 'model'. To the left of the equality sign the dummy argument R has to be entered. This argument channels the result of the model to the sum of squares routine. The right side has got to contain at least once the substrate or inhibitor concentration (S). During the fit the values for the (S) symbol are read from the source record with the experimental data. The model may use up to six parameters (C1 to C6) that are optimized so that the sum of the squares of the differences between the model and the experimental data become minimal. Besides these entries the model may contain any operators, constants, and other source records.

Before the fit is executed, the number of data points of the experimental data is compared to the number of data points of the record containing the noise. If they are not equal, no fit is performed. Once the equation has been entered, the interpreter fit (model 10) is treated like other models. A source record, a noise record, and a destination record for the simulated curve have to be selected. The first estimates together with the finest grid and the grid factors are entered. The fit is started with the 'execute fit' function in the fit menu. Sample outputs of the fit routine are listed in Figure 51.

APPENDIX

Appendix A

Glossary of Abbreviations and Symbols

A 1 Biochemical Abbreviations

c AAT	cytosolic aspartate amino transferase
m AAT	mitochondrial aspartate amino transferase
Bis-tris	N,N-bis(2-hydroxyethyl)amino tris(hydroxymethyl)-methane
BSA	bovine serum albumine
CD	circular dichroism
Chloramine-T (CT)	sodium-N-chlorotoluene-p-sulfonamide
CM(C)	carboxy methyl (cellulose)
DCCD	Dicyclohexylcarbodiimide
DEAE	diethylaminoethyl
DNA	desoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EPR	Electron proton resonance
MCD	magnetical circular dichroism
Page	polyacrylamide gel electrophoresis
SDS	sodiumdodecylsulfate
TLC	thin layer chromatography
TMPD	N,N,N,N-tetramethyl phenylene dimethyl dihydrochloride
Tris	tris(hydroxymethyl) aminomethane
UV	ultraviolet
Vis	visible
w/v	weight to volume ratio
ϵ	extinction coefficient in ($\text{mM}^{-1}\text{cm}^{-1}$)
$\Delta\epsilon$	CD ($\text{mM}^{-1}\text{cm}^{-1}$)
$\Delta\epsilon/H$	MCD ($\text{mM}^{-1}\text{cm}^{-1} \text{ T}^{-1}$)
$\Delta\epsilon$	absorption difference ($\text{mM}^{-1}\text{cm}^{-1}$)
$\Delta(\Delta\epsilon)$	CD difference ($\text{mM}^{-1}\text{cm}^{-1}$)
$\Delta(\Delta\epsilon/H)$	MCD difference ($\text{mM}^{-1}\text{cm}^{-1} \text{ T}^{-1}$)
R_s	hydrodynamic radius
M_r	relative molecular mass (dal)

A 2 Technical Abbreviations

ASCII	american standard code for information interchange
AU	absorption unit (=OD)
BASIC	beginners all purpose instruction code
CMOS	complementary metal oxide semiconductors
cr	carriage return (OD ₁₆ = 10 decimal)
CPU	central processing unit
Hex. (H)	hexadecimal
HP	Helwett Packard
IEC	international electronics commission
IEEE	institute of electrical and electronics engineer's standard
IO	input output
I	light intensity
I _o	light intensity of lamp
I _s	stray light
K _d	dissociation constant (μM)
K _i	inhibition constant (μM)
K _m	Michaelis constant (μM)
LCD	liquid cristal display
LED	light emitting diode
If	line feed (OF ₁₆ = 15 decimal)
NAND	not and gatter
NOR	not or gatter
OD	optical density (=AU)
RAM	random access memory
RIA	radio immuno assay
ROM	read only memory
TN _{max}	maximal turnover number (s^{-1})
TTL	transistor transistor logic
V _{max}	maximal velocity (U s^{-1}).
Z-80	Zilog 8-bit central processing unit

Appendix B

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Appendix C

Data Structures

C 1 BITRATE User Software

This section summarizes the data structures that are used in the BITRATE software.

a) Common data block of BITRATE user software

The common data block of the BITRATE software is a portion of RAM which is reserved in all subroutines. It is used to exchange spectra, measurement parameters, flags, and pointers between the subprograms. The entries in this block are listed in the table below :

S1(152)	I _O (emission spectrum of light source)
S2(152)	I _S (stray light spectrum)
S3(152)	spectrum of enzyme in absorption units
S5(152)	spectrum of buffer in absorption units
S4(20)	scratch buffer
T(20)	measurement parameters (see next page)
T1(82)	data of titration curve 1 in absorption units
T2(82)	data of titration curve 2 in absorption units
L1-L6	wavelengths ranges of titration curves (in nm)
D1\$(1) *	series of measurement
D2 *	number of measurement
D4\$(20) *	flags (blank correction)
E1\$(20) *	name of enzyme in cuvette
E2\$(20) *	name of ligand in syringe
T1\$(65) *	comment 64 characters
E1\$(20) *	label of X-Axis
E2\$(20) *	label of Y-Axis
D3\$(20)	date of measurement
B9\$(8)	name of volume (disc)
C0	position of stepper motor in pulses
Z5	wait after titration until measurement (s)
Z6	internal / external reference
Z7	I _O measurement valid or invalid
Z8	initiation flag
Z9	maximal absolute noise that will be tolerated
F8(5)	flags
D\$(20)	file names
Y0,Y1,Y2,Y3	active y-axis of plot and display

(*) header in measurement parameter file

b) Measurement parameter file

The measurement parameter files are generated by the 'Sto Pa' routine in the main menu of the BITRATE software. They are used to store the set up, conversion factors, and enzyme names. Such a file is generated for each different enzyme or ligand. The meaning of the entries is listed in the table on the next page :

C 2 MICHKIN User Software

This section summarizes the data structures that are used in the MICHKIN software.

a) Common data block of MICHKIN user software

The common data block of the MICHKIN software is a portion of RAM which is reserved in all subroutines. It is used to exchange spectra, measurement parameters, flags, and pointers between the subprograms. The entries in this block are listed in the table below :

S1(80)	time course 1 (absorption versus time data)
S2(80)	time course 2 do.
S3(80)	average of 1 do.
S4(10)	scratch buffer
T1(41)	titration curve 1 ($\Delta AU / s$)
T2(41)	titration curve 2 ($\Delta AU / s$)
D1\$(1) *	series of measurement
D2 *	number of measurement
D4\$(20) *	flags (blank correction)
E1\$(20) *	name of enzyme
E2\$(20) *	name of ligand
T1\$(65) *	comment (64 characters)
T(20) *	measurement parameters
L(20) *	do.
B(20) *	do.
W(20) *	syringe set up (volumes, speed)
W1(20) *	stopped flow commands
A(5,5) *	axes of plots
D3\$(20)	date of measurement
C(15)	counters of syringes (in ml)
E(20)	enzyme concentrations in autosampler (μM)
Z5-Z9	flags
B9\$(8)	name of volume (disc)

(*) stored in measurement parameter file

b) Measurement parameter file

The measurement parameter files are generated by the 'Sto Pa' routine in the main menu of the MICHKIN software. They are used to store the set up, conversion factors, and enzyme names. Such a file is generated for each different enzyme or substrate. The meaning of the entries is listed in the table on the next page :

(*) header of measurement parameter file as common data variables

T(1)	λ -start of curve 1	B(1)	maximal noise in %
T(2)	λ -stop of curve 1	B(2)	maximal nonlinearity in %
T(3)	enzyme conc. in nM	B(3)	actual enzyme conc. in assay
T(4)	substrate conc. in μM	B(4)	starting enzyme conc. (auto)
T(5)	no. of points	B(5)	preselect enzyme in assay
T(6)	start time of time win dow at max. substrate	B(6)	actual substrate conc. in assay
T(7)	stop time do.	B(7)	assay volume in ml
T(8)	number of repetitons	B(8)	
T(9)	data format	B(9)	
T(10)	λ -start for internal	B(10)	
T(11)	λ -stop reference	B(11)	measurement interval (s)
T(12)	λ -start range 2	B(12)	integration time (s)
T(13)	λ -stop range 2	B(13)	t-start of time window at
T(14)	[S] max. (μM)	B(14)	t-stop min. substr. conc.
T(15)	ϵ substrate range 1	B(15)	
T(16)	ϵ substrate range 2	B(16)	
T(17)	$\Delta\epsilon$ range 1	B(17)	
T(18)	$\Delta\epsilon$ range 2	B(18)	
T(19)	row type	B(19)	
T(20)	additional repetitions	B(20)	

c) Format of transmitted data

The MICHKIN software measures and stores velocities of enzyme reactions at different substrate concentrations (titration curves). These results are sent to the host preceded by a header (measurement parameters). The format of the transmitted data is :

1st. line : header

A..ldate.....flags.....enzyme name.....
substrate name.....comment (64 characters).

2nd line 20 variables

format : SDDDDD.DDDDD

3rd and fourth line optional variables with same format

data points in floating point format :

velocity 1, velocity 2, substate conc.

SD.DDDDEEEEE SD.DDDDEEEEE SD.DDDDEEEEE

d) Configuration of RS-232:

9600 baud

8 bit 2 stop bits

no parity

Xon/Xoff handshake

C 3 MICHFIT User Software

This section summarizes the data structures that are used in the MICHFIT software.

Common data block of MICHFIT user software

The common data block of the MICHFIT software is a reserved portion in RAM. It is used to exchange global variables between the subroutines. The global variables are explained in the table below :

Pointers and flags

pinit	initiation performed; this flag is set if the common data area has been initialized
forts	proceed pointer; this pointer is used to activate either input, content or output menu when the data processing subroutine is activated
tmod	transformation 1-6
tein	input device 1-6
taus	output device 1-6
teart	file type
idat	
name	name of user (used to identify workfile)
initialen	initials of user (used for file names)
pret	calling program is reactivated after termination of active subroutine
datstr	date generated by the main program is printed to curve fits and is shown by output menu
anzgem	No. to average
anztit	No. of curves in input and output menu
autoscale	autoscale flag on/off 1/0
interpolate	interpolate flag
anzeige	actual page in content menu
softk	active set of softkeys
offo%	roll state of output menu
offi%	roll state of input menu
format ()	prompts read from system files during start up
sym ()	symbols read from system files during start up and when other symbols are needed
inhalt ()	directory contains file names of 32 files of the same file type (e.g. kinetic files)
inhp()	pointer for number of entries of each file type in each drive (5 file types 5 drives)
macro ()	macros for command language
filename ()	file names are used to hand over file names to the input subroutine
iu()	screen update pointers; is used to store the parts of the menus that have to be updated if the computer is not busy.
ip()	workfile update; is used to store the records that have been changed by external routines.

10 data curves

psym(10)	symbols resp. line types (1-50)
stift*(10)	pen (0-8) for plotter and display
tque(20)	source registers in input and output menu
dmax(10)	pointers for the number of data points of each data curve in RAM
dx(10,332)	array of data curves
dy(10,332)	

definitions of plot

beschr ()	annotate strings, title, and labels of axes
tl(30)	string length
pg(20),pga(30)	plot definitions
pxu#(),pxo#()	minimum, maximum, delta x and delta xo
pyl#(),pyr#()	minimum, maximum, delta y and delta yo
pgx(),pgy()	sizes of title, labels, digits, symbols and annotate.
idx(),idy()	draw max 128 data points
idp()	pen, symbol, or line type

touchfields

icon()	definitions of touchfields
--------	----------------------------

C 4 MICHKIN System Software

Each AIOS or GIOS function is identified by a 16 bit function code. The function code and additional parameters have to be stored in a command buffer.

```
IN BUF    DW      1           ;function code
          DB      14          ;lower right column
          DB      16          ;lower right row
          DB      10          ;upper left column
          DB      15          ;upper left row
          DW      0FFFFH
          DW      0FFFFH
```

Then the processor registers have to be set up. The DS register has to contain the buffer's data segment.

```
DEFA      MOV      AX,4403H    ; I/O control write
          MOV      BX,1        ; console handle
          MOV      CX,10       ; buffer length
          MOV      DX,OFFSET IN_BUF ; offset address
          INT      21H         ; call function
          RET                  ; return
```

And the function is executed by an interrupt to the operating system.

The HP-150 provides a set of data communication I/O functions available in MS-DOS. The data comm functions are accessed in the same way as the AGIOS calls (by command buffer).

a) Touchscreen functions

The function FNTOUCH defines a field on the screen of the HP-150 with the upper left corner (row%,col%) and the size (rowinc%,colinc%), that generates the response (resp) when it is touched. Touching such a field is equivalent to pressing a key on the keyboard. The function OFFTOUCH removes all defined touchfields from the screen.

b) Controlling the alphanumeric screen

The alphanumeric screen can be cleared (CLS), switched on (ALPHA) and switched off (ALPHAOFF). The cursor can be located anywhere on the screen with the function CURS (col%,row%). One line of information can be written to the alpha memory or read from the alpha memory with the function READLI (H1) and DISLI (HS).

c) Read disc directory

Among the functions most necessary for a user-friendly software are the access to the disc directories. This is especially important when a large number of files from different users have been stored on a hard disc. The functions SUCHF and SUCHN are used to select only files that belong to a specific task. These files can be accessed subsequently simply by touching the fields in the INPUT menu.

d) Access to system clock

Two functions are provided to read the internal clock:

The function DATUM (year%,day%,dayofweek%) returns three 16 bit integers. The first integer is the year. The higher 8 bits of the second integer is the month, and the lower 8 bits the day of this month. The third integer returns the day of the week (1 - 7) starting from Sunday. The function ZEIT (minute%,second%) returns the hour of the day (0 - 24) in the upper half of the first integer and the minutes in the lower half. The upper half of the second integer contains the seconds and the lower half contains the hundredth of seconds.

e) Multiple screens

The STOSCR (#) function is used to store the contents of the alpha screen to a random access file on a mass storage medium, preferably a RAM disc or a hard disc.

With the RECSCR (#) function stored screens are recalled from a random access file that has already been opened. Only the first 21 lines of the screen are stored. The only limitation to the number of screens is the storage space and the speed of access.

f) Data transfer

Function POPEN (handle%) opens the serial port. It can then be treated as a character device from DOS. Function PCLOSE (handle%) closes the serial port. Function INSTAT (status%) checks if a byte is ready for input from the serial port. Function OUTSTAT (status%) checks if the serial port is ready to accept a byte.

g) Access to graphic functions

The graphic screen as well as the alpha screen can be controlled independently. The HP-150 uses a monochrome graphic screen with a resolution of 640 x 390 pixels. The functions switching the graphic screen on and off are GRAPH and GRAPHOFF. All entries on the graphic screen are removed by the GCLEAR function. Similarly to the alpha screen, several routines are used to control the graphic cursor. GCURS turns the graphic cursor on and GCURSOFF removes it. It can be moved absolutely to a pixel MGCURS (x%,y%) where x has to be in the range of 0 to 640 and y has to be in the range of 0 to 390.

Lines are drawn using the functions PLOTD (x%,y%) and PLOTU (x%,y%). X% and y% have to be in the same range as in the case of the graphic cursor. Lines can be drawn with different line types (0-10; see HP-150 Manual).

It is possible to write text to the graphic screen of the HP-150. The size, the direction, and the slant can be defined with the functions SIZE(x%), DIR(x%), and SLANT(x%) respectively.

Appendix D

Alphabetical List of Assayomate Functions

The System software of the Assayomate implements a comprehensive set of single key commands. They are activated by pressing the respective key on the keyboard of the Assayomate or by sending a command character with the IEC interface. The meaning of the commands is listed in the table below :

A	ALTM	<i>This function changes the volume that is moved by the functions B(give out), C(suck in), and D(give back) of the active syringe. The message displayed is 'PLEASE ENTER THE VOLUME IN (ML)', it expects the input of a number between 0 and the maximal volume.</i>
B	GIVE	<i>This function moves the volume defined by function A towards the mixing chamber and the measure head immediately. It displays 'GIVE OUT' during the operation.</i>
C	SUCK	<i>This function sucks the volume defined by function A from the reservoir into the syringe immediately. It displays 'SUCK IN' during the operation.</i>
D	BACK	<i>This function moves the volume defined by function A back to the reservoir immediately. It displays 'GIVE BACK' during the operation.</i>
E	WASH	<i>This function washes the active syringe by repetitive suck in and give out cycles. It displays 'PLEASE ENTER THE NUMBER OF CYCLES' and expects the input of a positive number in the range from 0 to 99. The first cycle empties the syringe, the next cycle sucks in 2% of the maximal volume, the third empties the syringe and so on. An odd number of cycles, stops washing with an empty syringe, an even number stops washing with a filled syringe. The message 'WASHING OF ACTIVE SYRINGE' is displayed during the entire operation.</i>
F	EMPTY	<i>This function empties the syringe back to the reservoir immediately, it displays the message 'EMPTYING SYRINGE' during the operation.</i>
G	FILL	<i>This function fills the syringe from the reservoir to the level entered by the function '=' immediately, it displays the message 'FILLING SYRINGE' during the operation. If the fill level of the syringe is already higher than the maximal fill level, the command is ignored.</i>
H	STOPFL	<i>This function mixes an assay, and displays the message 'STOPPED FLOW'. The amounts of buffer, enzyme and substrate have to be defined by the function 'K' before this function is executed. If one of the syringes runs out of volume, the function is immediately aborted. Approximately 100 milliseconds before the assay is finished, the busy bit in the status byte of the IEC interface is cleared, and after the termination of the assay, the menu is updated.</i>
I	GETNP	<i>This function moves the autosampler up, then changes to the next probe, and lowers the autosampler. During the operation the message 'GET NEXT PROBE' is displayed. The 'Next probe ready' bit is set in the status byte. If the door is open, if the autosampler does not move up, if the stop switch of the Assayomate is actuated, or if the tray is not in place, the function is ignored and an error message is displayed.</i>
J	TURNB	<i>This function withdraws the autosampler tip and turns the probe tray back to its start position. Moving the tray is refused, if it is not properly inserted, if the door is open, or if the autosampler can't move up. This command displays the message 'TURNING TRAY BACK' during the operation.</i>
K	SETSF	<i>This function is used to change the composition of the assay. It displays 'PLEASE ENTER THREE VOLUMES (ML)' and expects three parameters, the volume of buffer (in ml), the volume of enzyme (in ml), and the volume of substrate (in ml) e.g. 0.300,0.050,0.050(cr) This function does not execute the assay, this is done by function 'H'.</i>

L	FUVOL	This function furnishes the volumes of a series of assays to the display or the IEC listener depending on the source of the command. It displays 'VOLUMES OF SUBSTRATE' and then according to the setting of the number of points variable <i>n</i> times a volume e.g. 7 0.367(cr)(lf). The listener has to receive <i>n</i> lines of information.
M	SETRE	This function is used to set the type of substrate spacing, the number of points, the number of repetitions, the volume of the assay (in ml), the amount of enzyme (in ml), and the maximal amount of substrate (in ml) e.g. *M 1,20,3,0.400,0.040,0.100
N	FUBED	This function calculates the need of buffer, enzyme, and substrate according to the settings of function 'M'. Then it executes a fill command for all syringes, but only the syringes containing not enough volume are actually moved. If a syringe has to be moved, it displays 'FILLING SYRINGE'.
O	SFSET	This function is used to set the volumes for an assay by specifying which point of the active rowtype and number of points shall be prepared. Before this function can be executed, proper parameters for function 'M' have to be entered. It prompts 'PLEASE ENTER POINT (ASSAY)' and expects an integer in the range from 0 to the maximal number of points.
P	MSTAT	This function reads the status of the microswitches and copies it to the status byte.
Q	FINI	This function washes all syringes and prompts 'PLEASE SWITCH OFF ASSAYOMATE'. For this purpose, the reservoirs have to be filled with water before this function is executed. It empties and washes all syringes, first the substrate syringe, then the enzyme syringe, and finally the buffer syringe.
R	SWREM	Switches the Assayomate to remote control, after this function has been executed commands from the keyboard are no longer accepted. The only exception are the screen roll and the LOCAL function. It displays 'SWITCHED TO REMOTE'.
S	DIHEME	This function prompts 'PLEASE ENTER HEXADECIMAL ADDRESS' and expects a hexadecimal number in the range of 0 to FFFF. It displays 8 bytes of memory in hexadecimal starting with the memory location that was entered. *S F800
T	LDHEME	This function prompts 'PLEASE ENTER HEXADECIMAL ADDRESS' and expects a hexadecimal number in the range of 0 to FFFF. The hexadecimal address can be followed by to 32 pairs of hexadecimal numbers each separated by a space from the next pair. This function converts the pairs of hexadecimal numbers to binary (0-255) and stores them to the Assayomate memory. *T F800 AA CF 01(cr)
U	LOCAL	switches to local (commands from keyboard accepted) It displays 'SWITCHED TO LOCAL'.
V	REASC	This function expects a hexadecimal address and a string of ASCII characters from the IEC talker. The ASCII string is stored to the Assayomate memory.
W	SEASC	This function expects a hexadecimal address and sends the content of the Assayomate memory to the IEC listener until a carriage return is encountered. The longest string that will be sent is 128 Bytes. If the IEC listener is not ready to receive data the Assayomate displays 'IEC TIMEOUT' after 1 second and aborts the operation.
X	SPEED	This function is used to change the speed of piston movement of the active syringe. It displays 'PLEASE ENTER THE SPEED IN ML/MIN' and expects the input of one number in the range from 0 to 99 ml/min.
Z	NEWFU	This command executes the RAM based routine that has been loaded to the memory of the Assayomate before.
=	LEVEL	This function prompts 'PLEASE ENTER FILL LEVEL IN ML' and expects one number in the range from 0 to the total volume of the active syringe.
0	NOSTIR	Switches off the stirrer motor.
1	AKTA	Keys 1, 2, and 3 activates the buffer, the enzyme, and the substrate syringe, respectively. Key 5 activates the tray and key 6 activates autosampler.
9	NEXTP	This function sets the 'next probe ready' bit in the status byte.

Appendix E

Electronic Scheme of Binding Titrator

The HP8451A uses an IEC bus to transfer data to disc drive, printer and plotter. The same bus line can be used to control up to 15 peripheral devices. In addition to the 8 data lines, there are several control lines one of which, the REN line, is not used in this configuration (see Table 12). For this reason, the REN line was used to activate (enable) the stepper motor controller.

Controller

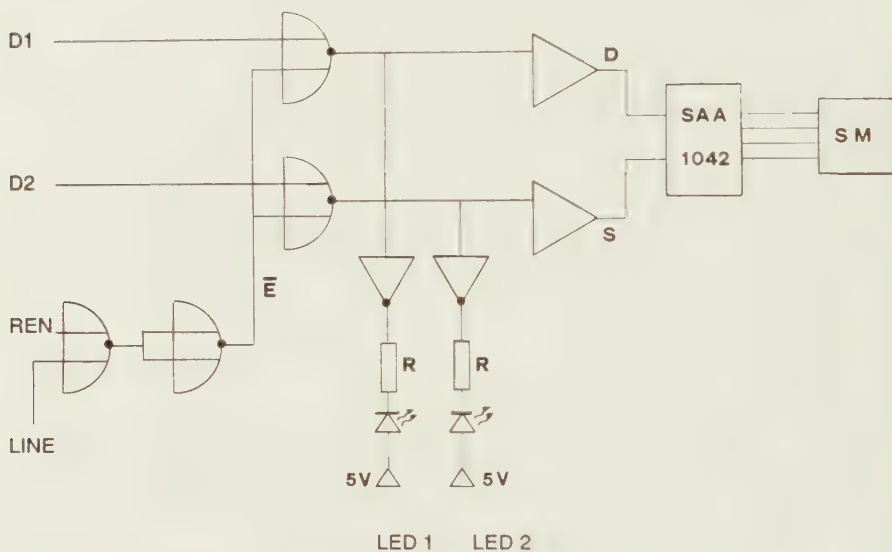
Data line 1 controls the direction (R) of the stepper motor. A step is executed when a short pulse (S₁) is sent on data line 0.

Note : IEEE standard 488 specifies that the bus lines use negative true logic. All lines are shown in the schematic with bars over them. Thus, if a bus line is true, it will have a low voltage level. Each line has driver receiver circuits with the following characteristics :

State	drivers	receivers
Low state :	$\leq 0.5 \text{ V}$	$\leq 0.8 \text{ V}$
High state :	$\geq 2.5 \text{ V}$	$\geq 2.0 \text{ V}$

It is possible to connect the bus lines without a transceiver to TTL (LS) gatters. It is important to set the REN line to true only when the stepper motor is moved. Immediately after the move and after a hard reset, this line has to be set to false (LOCAL 7), otherwise address information and data transfer to other devices move the stepper motor. The stepper motor controller can be disconnected logically from the bus by switching the 'line' switch to the 'off line' position.

The controller needs two power supplies; +5 V (200 mA) for the logical circuits and +12 V (1 A) for the operation amplifiers (μA 747 CN) and for the stepper motor driver. The status of the (enabled) bus lines is displayed by 8 LED's driven by NOT gatters in the low state, the current is adjusted by a 1 K Ω resistor.



D = direction S = step E = enable SAA 1042 = stepper motor controller SM = stepper motor R = resistor D1 = data line 1 D2 = data line 2 REN = remote enable LINE = switch on controller front panel (on line / off line)

Figure 54: Electronic scheme of binding titrator

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